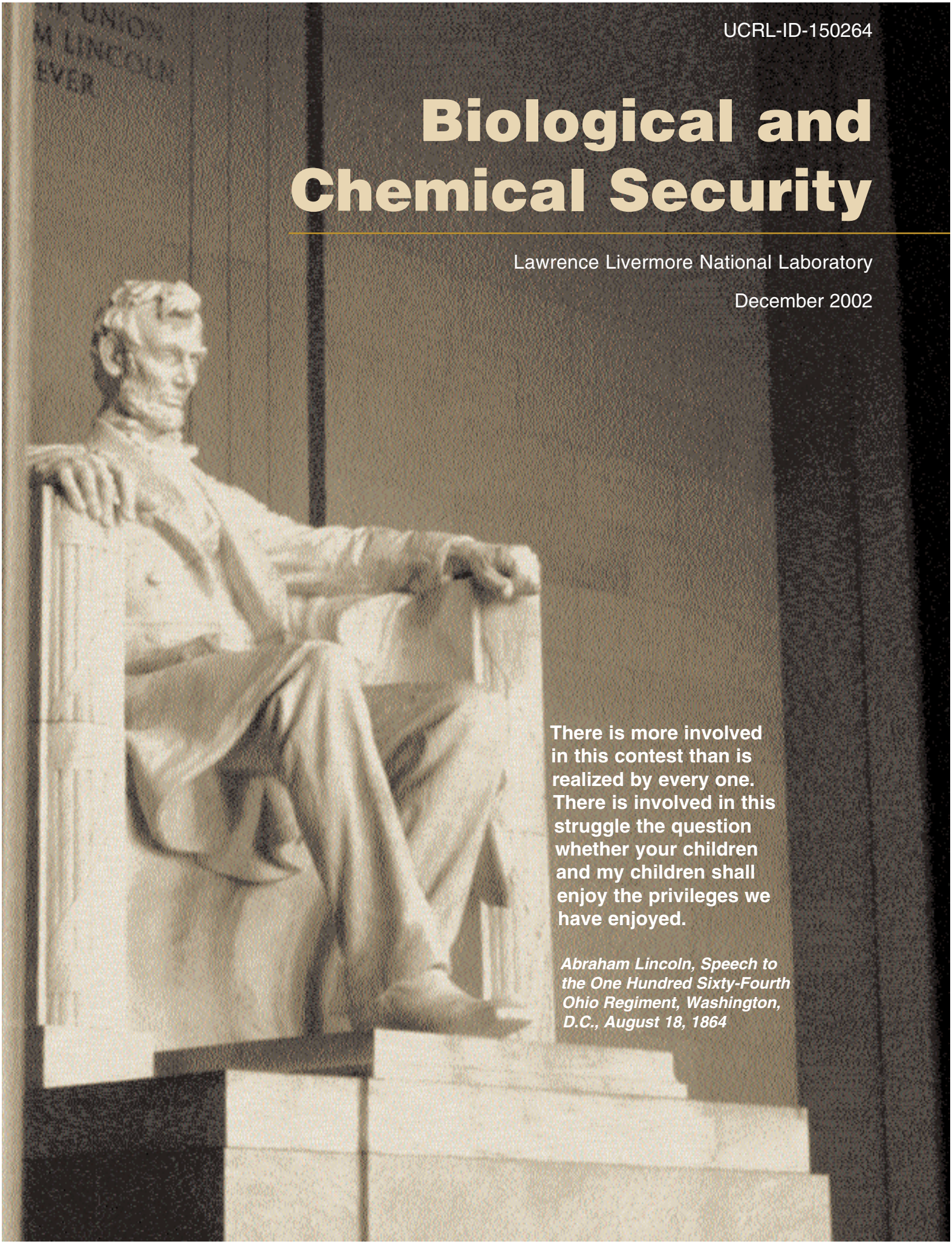
UCRL-ID-150264

Biological and Chemical Security

Lawrence Livermore National Laboratory

December 2002



There is more involved in this contest than is realized by every one. There is involved in this struggle the question whether your children and my children shall enjoy the privileges we have enjoyed.

Abraham Lincoln, Speech to the One Hundred Sixty-Fourth Ohio Regiment, Washington, D.C., August 18, 1864

Prepared by **LLNL** under contract
No. W-7405-Eng-48

Reprinted from

Science & Technology REVIEW

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Biological and Chemical Security

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Chemical & Biological National Security Program

The LLNL Chemical & Biological National Security Program (CBNP) provides science, technology and integrated systems for chemical and biological security. Our approach is to develop and field advanced strategies that dramatically improve the nation's capabilities to prevent, prepare for, detect, and respond to terrorist use of chemical or biological weapons.

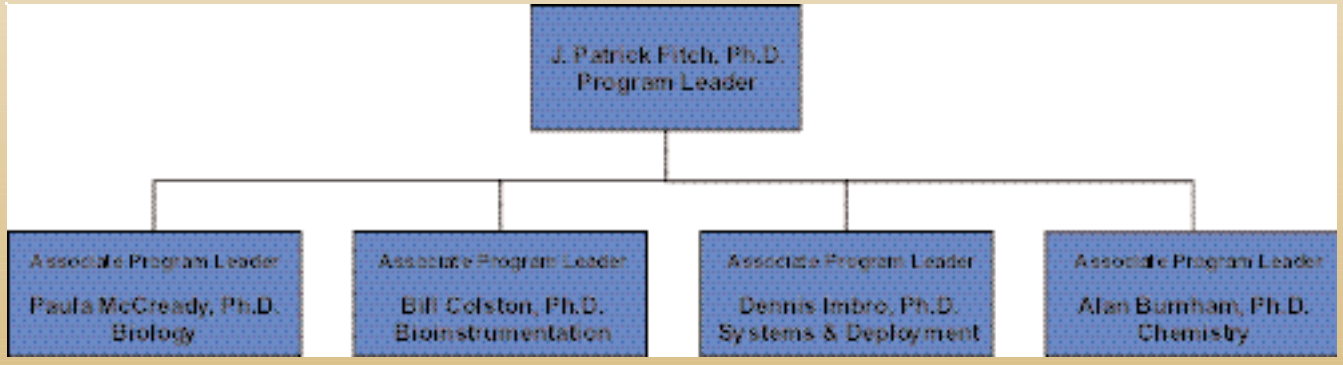
Recent events show the importance of civilian defense against terrorism. The 1995 nerve gas attack in Tokyo's subway served to catalyze and focus the early LLNL program on civilian counter terrorism. In the same year, LLNL began CBNP using Laboratory-Directed R&D investments and a focus on biodetection. The Nunn-Lugar-Domenici Defense Against Weapons of Mass Destruction Act, passed in 1996, initiated a number of U.S. nonproliferation and counter-terrorism programs including the DOE (now NNSA) Chemical and Biological Nonproliferation Program (also known as CBNP). In 2002, the Department of Homeland Security was formed. The NNSA CBNP and many of the LLNL CBNP activities are being transferred as the new Department becomes operational.

LLNL has a long history in national security including nonproliferation of weapons of mass destruction. In biology, LLNL had a key role in starting and implementing the Human Genome Project and, more recently, the Microbial Genome Program. LLNL has over 1,000 scientists and engineers with relevant expertise in biology, chemistry, decontamination, instrumentation, microtechnologies, atmospheric modeling, and field experimentation. Over 150 LLNL scientists and engineers work full time on chemical and biological national security projects.

Critical shortfalls exist in our ability to prevent, prepare for, detect, and respond to chemical or biological terrorism.

LLNL CBNP is developed and executed by a multidisciplinary senior management team

Key assignments (partial list)



NNSA

- Biofoundations Coordinator: Bert Weinstein
- Modeling & Prediction Coordinator: Don Ermak

LLNL

- Decontamination: Ellen Raber
- Modeling & Prediction: Gayle Sugiyama
- Forensic Science Center: Glenn Fox
- Pathomics: Fred Milanovich
- Informatics: Tom Slezak
- BBRP: Ken Turteltaub
- EE: Betsy Cantwell
- ME: Ravi Upadye
- CMS: Al Ramponi

The LLNL CBNP emphasizes collaborative demonstration programs with end-users as part of a spiral development strategy. These programs provide near-term capabilities, opportunities for dialogue among scientists, engineers and end-users, focus for R&D investments, and paths for pilot-to-regional-to-national systems.

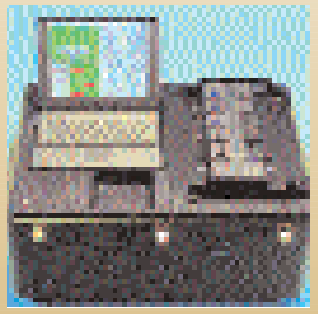
Current and emerging collaborative demonstration programs include

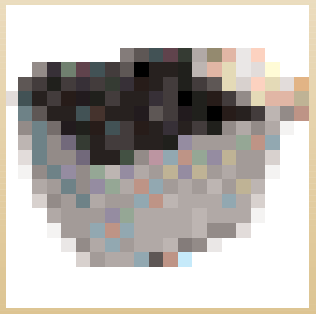
- Biological Aerosol Sentry and Information System (BASIS) is a "detect to treat" environmental monitoring system originally designed for the Salt Lake City 2002 Winter Olympics. BASIS is a joint project of Lawrence Livermore and Los Alamos National Laboratories with significant participation by law enforcement and public health organizations. BASIS successfully deployed to the Olympics and has also been deployed to several other sites. In its first year of continuous operation, it has performed over 70,000 diagnostic tests of complex environmental samples with no false alarms.
- Laboratory testing of sentinel populations for early detection of bioterrorism (SENTINEL) complements BASIS-type environmental monitoring by performing surveillance of the population through direct high-throughput testing of clinical samples of opportunity. LLNL has demonstrated 1,000 samples (10,000 assays) performed in an 8-hour shift with 2 technicians. SENTINEL is an emerging program with the influenza-like-illness programs and the Veterans Administration to directly detect pathogens and toxins in samples. Preliminary data by LLNL and its corporate collaborator Source Precision Medicine, indicates that the strategy might also be applied to presymptomatic detection of host response biomarkers.
- Local Integration of NARAC in Cities (LINC) provides responders and local government with modeling and prediction tools for decision support. Composite views of plume prediction with important local features including schools and fire and police stations are available to the users. Both local and reach-back capabilities are provided. The first pilot city for LINC evaluation began in September 2002 and is Seattle, Washington.
- Bio-Forensics is a joint LLNL, Northern Arizona University, and Los Alamos National Laboratory project to make specific forensic tools and data available to the

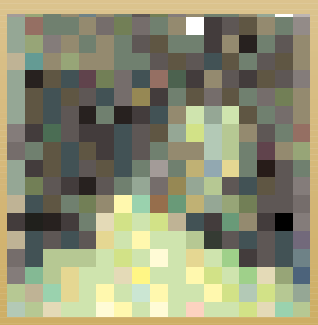
Advanced Nucleic Acid Analyser

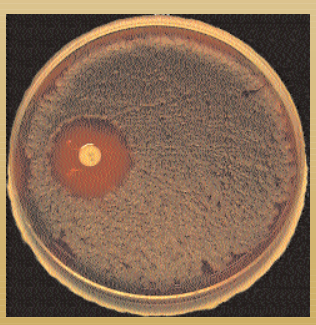
The LLNL Advanced Nucleic Acid Analyzer demonstrated 7 minute PCR detection in April 1999. LLNL technology licensed to Cepheid helped create the Smartcycler product that is now used in the LLNL field lab.

LLNL restoration R&D ranges from development of new decontamination agents, to room-size demonstrations, to evaluation of large-scale restoration for special facilities like transportation hubs.







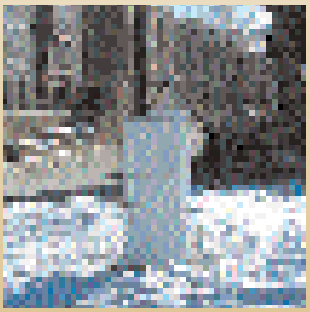


broader community. The focus is on supporting law enforcement and the CDC PulseNet Laboratories for food borne illnesses. Round robin comparisons of different methods and assays will precede the principle deliverables of 1) a database for a variety of end-users and focused on law enforcement and 2) strain-sensitive markers and validated assays for *Salmonella* and *E. coli* for CDC PulseNet.

- PROTECT is an Argonne, Sandia and Lawrence Livermore National Laboratories program for protection of key facilities focused mostly on chemical attacks. LLNL is supporting PROTECT through NARAC and biodetection. NARAC is doing modeling and predictions for outside facility and scenario studies including operational support of deployed systems.
- OPCW Certified Laboratory is in support of the US State Department's selection of LLNL as the second US designated laboratory for the Office for the Prohibition of Chemical Weapons (OPCW). We have implemented all the technical, safety and procedural systems required and been ISO-17025 approved. Final OPCW designation is pending. The first, and currently only, US OPCW Certified Laboratory is at ECBC in Edgewood, Maryland. The ECBC group has provided significant assistance to LLNL in this process.
- Restoration of Operations will demonstrate strategies for decontamination and restoration of operations for major transportation facilities. The activity builds on LLNL restoration planning for CW attacks on transportation facilities, decontaminant reagents including L-gel, sampling strategies to support decontamination, high throughput sample processing, accelerated viability testing, and published studies to establish "How clean is clean enough?" Lawrence Livermore and Sandia National Laboratories jointly execute the project with a focus on airports and establishing "templates" for restoration strategies.
- Model Cities is a tri-Lab project to better understand geographic and other local or regional factors that influence prevention, preparation and response. The goal is to create common "templates" that can be applied in many locations and in combination to create more comprehensive regional plans. The initial test beds for this concept have been the other demonstration projects (e.g., BASIS and

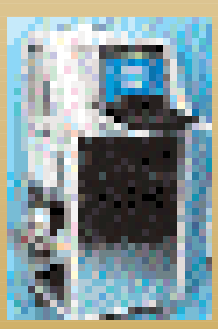
BASIS Biodetection System

The BASIS biodetection system is based on a aerosol collector network with a central deployable field laboratory. The field lab can also support decontamination/restoration activities and clinical sentinels for early detection. In December 2002, over 10,000 assays were performed in an 8 hour shift by two technicians. A next generation instrument (APDS) is in development by LLNL and includes the collector and laboratory in one unit with unattended operation for several days. Miniaturization of various components improve performance and reduce costs.



Collector

Laboratory



APDS

Future

LINC) and a tri-lab demonstration in Albuquerque, New Mexico in December 2002. Integration and evaluation of multiple detection schemes for wide area, special facility, and epidemiological surveillance were accomplished.

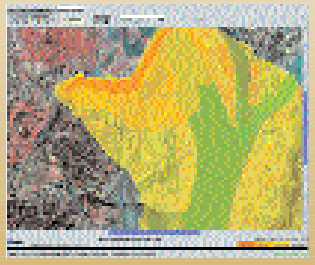
The demonstration programs help focus our science and technology (S&T) investments. The S&T is managed in four areas 1) Decontamination and restoration; 2) Modeling and prediction; 3) Instrumentation; and 4) Applied science. Some of the current S&T activities include

- **Decontamination and restoration** goal is to provide the S&T and systems approach to quickly restore civilian facilities to operation. Decontamination in a civilian setting requires fundamentally different technology from most military applications. Rapid and effective means of decontamination are needed for equipment, facilities and large urban areas. LLNL developed the L-gel decontamination technology. Several new decontamination chemistries are in development and testing including vaporous hydrogen peroxide. In addition, appropriate protocols for efficient restoration are being investigated.
- **Modeling and Prediction** goal is to develop predictive urban environment modeling tools for local and other users for response, planning and vulnerability assessments. Advances in computing algorithms and hardware now make it possible to model air flows over very complex terrain. LLNL is developing tools for modeling such flows in urban environments including around buildings and in subways to determine in advance how to best respond and to permit real-time prediction of agent dispersal during an actual event.
- **Instrumentation for Chemical and Biological Detection** goal is to provide highly sensitive and accurate instruments for early warning, treatment triage, and detection of contaminated areas. LLNL is developing and integrating instruments with substantial increases in detection performance. The focus of chemical instrumentation is miniaturization of laboratory-quality instruments for field detection of CW agents and related material. LLNL has licensed several chemical instruments to industry including miniature GC-MS, thin layer chromotagraphy, and an integrated optic capillary electrophoresis system. LLNL technology for rapid DNA detection was licensed to industry and enabled a successful product that has been applied to counter terrorism. LLNL has prototyped a new instrument that is capable of 100 simultaneous assays; detection of viruses, toxins, spores, and vegetative bacteria; and capable of autonomous operation for several days at a time. Prototypes of this instrument have

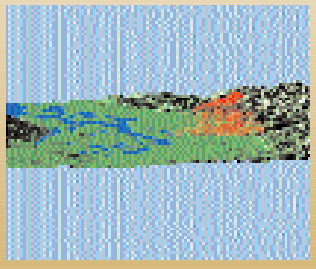
Atmospheric Modeling

Atmospheric modeling and experiments are being used to assess sensor placements and to provide feedback to first responders in urban environments. Photograph of August 7, 1998 tire fire in Tracy, CA with LLNL/NARAC-simulated particle positions in red (top) and city-wide simulations of a plume in Washington, DC amd Seattle, WA.

Tracy



Washington, DC



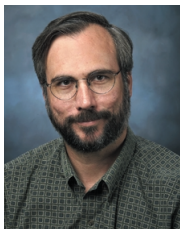
Seattle

been tested with aerosolized live agents and were deployed in a limited capacity in 2002. LLNL has licensed this technology and will assist our corporate partner to enable and accelerate access to this technology as a commercial product. Several next generation instruments are part of our R&D portfolio and address significant current shortcomings including cost per assay, operational complexity, and real time response.

- **Applied science** goal is to provide biological and chemical support for detection and other countermeasures. LLNL invented and transferred to industry specialized solid-phase absorbants that greatly simplify field collections of CW-associated materials. The availability of DNA and RNA sequence information has enabled rapid development of biological signatures that are highly specific and sensitive. Techniques are also being developed for ligand signature discovery as well as sub-species level bio-forensics. LLNL has invented several high throughput approaches for vetting signatures (both nucleic acid and ligand) including computational screening of potential signatures, automated strain panel testing, complex environmental sample testing and, more recently, pathogen-associated function and host-associated response using genomic and proteomic tools.

In the following pages, the reader will find a series of Science & Technology Review reprints representative of CBNP activities. Many of the point of contacts have changed—feel free to contact members of our team or me for additional information. The LLNL CBNP is organized into four areas managed by Associate Program Leaders. Because of the wide range of expertise and facilities needed to fulfill our mission in Chemical and Biological National Security, the CBNP also relies on a multi-disciplinary team with a number of key assignments.

Abraham Lincoln faced significant challenges to our homeland. His words remain powerful and, unfortunately, relevant today. Our program is fortunate to have a team willing to commit their careers and more to help our country meet today's challenges and preserve what we all hold so dear.



J. Patrick Fitch, Ph. D.
Program Leader
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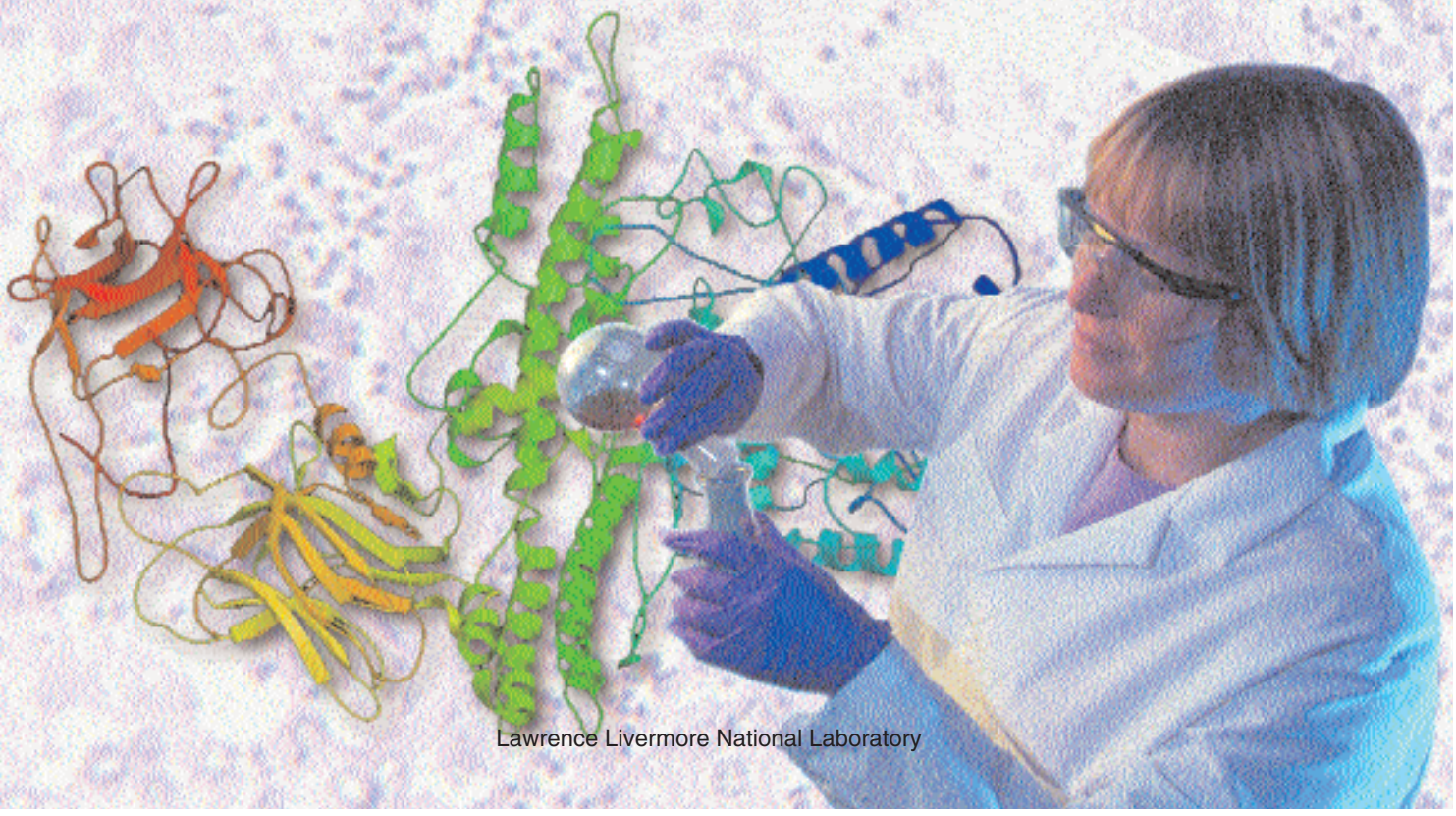
A Two-Pronged Attack on Bioterrorism

Livermore scientists are designing tiny synthetic molecules to detect biological warfare agents and fight cancer.

NEWLY designed molecules that bind to and capture biowarfare agents are on the drawing board at Livermore. The goal is for these molecules to quickly and efficiently detect such deadly pathogens as botulinum toxin, anthrax spores, or smallpox. Using synthetic chemistry, scientists produce these new molecules that bind to unique sites on the surface of the toxin or organism. Their two-pronged, or bidentate, structure is critical. When a small molecule binds to a protein, the attachment is usually weak, and the interaction between the two is short-lived. If, however, two or more small molecules that bind to the protein are linked together, their binding to the same protein may be thousands, even millions, of times stronger. By targeting specific proteins, the synthetic molecules will mimic some of the behavior in our immune system where antibodies recognize molecular foreign entities in our bodies and abnormalities such as cancer cells.

A single detector armed with many of these synthetic targeting molecules could simultaneously recognize an equal number of harmful biological agents that might be used in a terrorist attack. Assays using antibodies, known as immunoassays, are widely used to identify pathogens in the laboratory and form the basis for many biowarfare



Lawrence Livermore National Laboratory

detection systems fielded to date. However, only seven good antibodies are currently available for pathogen detection. Other detectors depend on recognizing the bioagent's DNA. "But some pathogens, such as viruses, require human exposure to only a small number of organisms to be acutely toxic," says Livermore biochemist Rod Balhorn. "With so little DNA present in each virus and given the rapid variation that occurs in the base sequences that make up the DNA, those pathogens are typically very difficult to detect."

Similarly designed targeting molecules could zero in on defective or overactive proteins in our bodies and poison them, just as our natural antibodies do. These antibodylike molecules can lock on to cancer cells or other pathogens and kill them—and only them. By targeting unique sites on other proteins that cause disease—for example, the proteases that cause inflammation in arthritis or enable HIV to function—the synthetic molecules would block the activity of the protein without entering its active site. The active site is a cavity on the surface of a protein that is used by the protein to perform its function. Similar active sites can be present in many proteins, both those that are essential to cell function and others that cause disease.

The pharmaceutical industry has already begun using this approach to develop drugs that function as intended without blocking the activity of healthy cells or proteins. Molecules that target unique sites on the surfaces of specific proteins may soon lead to a new generation of drugs that have minimal side effects.

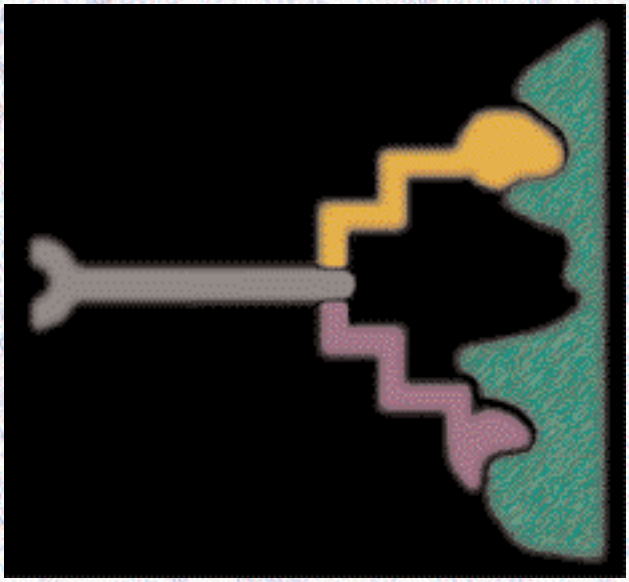
Balhorn is leading the program at Livermore to design synthetic molecules for bioagent detection and cancer treatment. He and a team of Livermore investigators are collaborating with scientists at Brookhaven and Sandia national laboratories and the University of California at Davis Cancer Center. Together, they are developing the methods needed to produce the first of these synthetic antibodylike molecules.

"Terminology is a little tricky," he notes. "It is tempting to call our new molecules 'synthetic antibodies.' But we are designing small molecules that function like antibodies, not large proteins that are synthetic versions of antibodies. So we use the term 'high-affinity ligands' to describe our molecules."

"Ligand" is a general term used to describe a small molecule that binds to proteins or other large molecules. The higher the affinity a ligand has for a specific protein, the more tightly it binds to it. Research by others has demonstrated that bidentate ligands have a vastly increased affinity for the target protein, anywhere from thousands to millions of times greater. Polyvalent ligands—molecules that bind to multiple sites on the surface of a protein—are observed in many biological interactions that require very tight binding. The seek-and-destroy antibodies of our immune system, which normally operate quite successfully, are one example.

"What we're doing is searching for two molecules that bind to two sites next to each other on the surface of a protein," says Balhorn. "Then our synthetic chemist joins them together using a third molecule, called a linker. The linker must be both flexible and robust, or the new molecule will fall apart. This new synthetic ligand will then behave pretty much like an antibody, binding tightly to the protein."

The new bidentate molecules, called high-affinity ligands (HALs), will have several advantages over naturally occurring antibodies. They can be totally inorganic (nonprotein) and can be synthesized in large quantities using methods to ensure that each batch is structurally and functionally identical. They will also be stable over a long period, making them excellent candidates for long-term deployment in detectors for agents of biological warfare.



This schematic diagram shows how a linker molecule will connect molecules that bind to two sites on a protein. The goal is to develop a process for designing and producing high-affinity ligands for any structured surface. When two molecules are connected with a linker, they bind with up to a million times higher affinity than does each molecule alone.

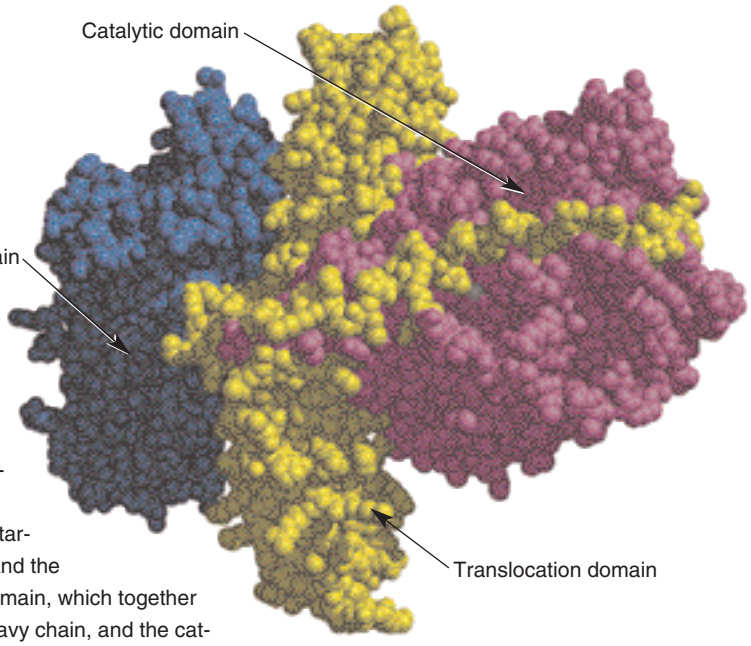
The Toxic Targets

As bioagent detectors, HALs can be designed to target protein toxins produced by pathogens as well as any major protein component of pathogenic organisms. For the National Nuclear Security Administration's Chemical and Biological National Security Program, work is under way to develop HALs that bind to the *Clostridium* neurotoxins, which include botulinum and tetanus, the most toxic substances known. The *Clostridium* toxins attack the central nervous system and cause spastic paralysis in the case of tetanus and flaccid paralysis in the case of botulinum.

Balhorn's team is laying the groundwork for future development of HALs to target the *Staphylococcus* enterotoxins, which cause acute intestinal symptoms such as those associated with food poisoning, and ricin, a residue of castor bean processing that causes major intestinal or respiratory complications. The body's response to toxic quantities of either of these substances is swift and often fatal.

Work is also scheduled to begin in the near future on HALs that bind to proteins in the spores of *Bacillus anthracis* (anthrax) and in *Yersinia pestis* (plague). Once these HALs are completed, efforts will focus on the next highest priority agents: smallpox, *Francisella tularensis* (a plaguelike illness), and *Brucella melitensis* (an organism whose infections, often called Mediterranean fever, cause spontaneous abortions). Creating synthetic ligands even for proteins with a known structure is still a research project. Work began in 2000, and Balhorn estimates that high-affinity ligands for these eight bacterial toxins and threat organisms can be delivered in about 2005.

Got Structure?



Clostridium neurotoxins have three parts: the targeting domain and the translocation domain, which together make up the heavy chain, and the catalytic domain, which makes up the light chain and is the toxic part of the molecule.

If the structure of the target protein is known, the team uses that structure to develop a HAL. Work on these molecules is a logical progression from Livermore's protein structure and computational biology effort, with which Balhorn has been involved since its inception. (See *S&TR*, April 1999, pp. 4–9.) Using x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, high-resolution structures for many proteins have been determined at laboratories around the world, including Livermore. These include several types of *Clostridium* toxins (botulinum and tetanus) and the *Staphylococcus* enterotoxins.

All toxins in the *Clostridium* family have three parts. The targeting (or binding) domain, which binds to receptor molecules on the nerve cell membrane, and the translocation domain, which makes a pore in the cell through which the toxin passes, together make up what is known as the heavy chain. The light chain, which contains the catalytic domain, is a protease that is injected into the nerve cell and disrupts its functioning.

For the *Clostridium* neurotoxins, the team is developing a HAL to bind to the targeting domain, that fragment of the protein that recognizes and binds to motor neurons. Of these neurotoxins, botulinum is considered a greater threat than tetanus, but tetanus is easier to work with. Fortunately, its targeting domain is sufficiently similar in structure to botulinum's that it serves as a model for botulinum.

In 1998, Livermore's x-ray crystallography group completed a high-resolution structure of the binding domain of the tetanus toxin. Researchers then computationally calculated the molecular surface of the protein to identify sites where binding is likely to occur. "We look for pockets on the surface of the folded protein,

places where another molecule would be able to fit tightly," says computational chemist Felice Lightstone. For the tetanus toxin, Lightstone found two appropriate sites adjacent to one another on the binding domain.

For a HAL to be effective, the sites designated for binding must be on a part of the toxin that is "conserved," meaning that these regions remain essentially identical across all strains of a toxin. When bioagents are being genetically engineered, areas such as these are difficult to modify without altering the toxicity of the agent. Ideally, a high-affinity ligand for tetanus toxin will be able to recognize engineered and other unknown or related *Clostridium* toxins.

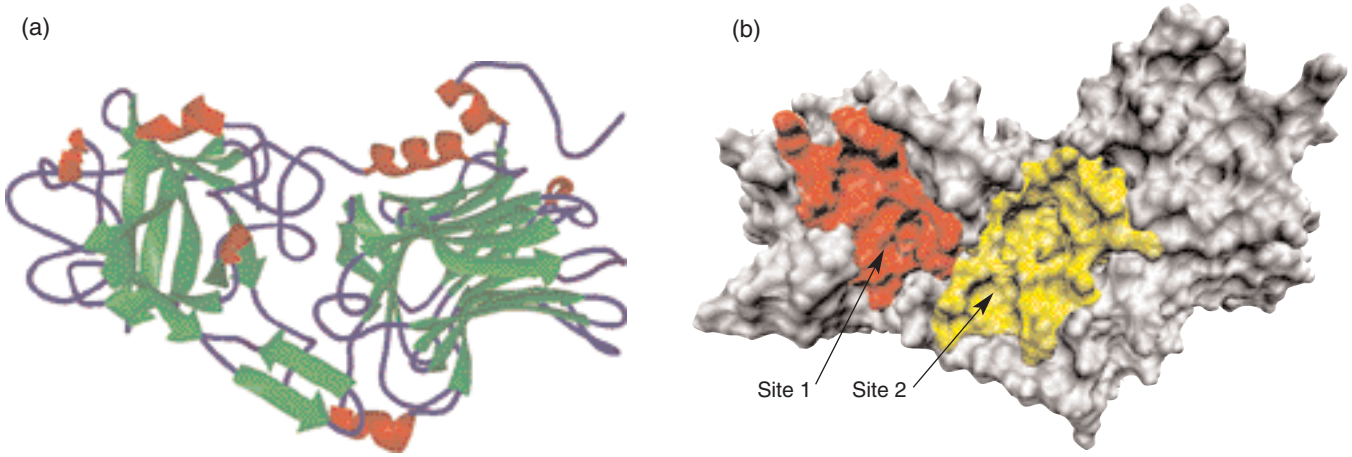
The next step involved selecting compounds that might fit into the two sites. All of the 300,000 compounds in the Available Chemicals Database, a listing of all commercially available compounds, were computationally inserted (docked) into each site. The potential fit and interactions were then assessed. The top 1,000 compounds were run again using a range of structures for each compound representing the different bond orientations and shapes, known as conformations, that each molecule is likely to adopt. In this manner, the top 100 compounds were identified. The calculations for each site took about 3 weeks on a Linux cluster of 40 dual-processor personal computers.

Sandia National Laboratories in Livermore has recently written new programs to expedite this time-consuming process. Each compound is tested in 10 different conformations to see which fits best into the rigid protein. This provides a more realistic test of binding, because many of these small molecules are not rigid and can adopt different conformations. "Computational docking projects typically have success rates of anywhere from 10 to 40 percent," says Lightstone. "Even before we started using our new version of this program, our success rate of identifying molecules that actually bind to the protein was in the 40- to 65-percent range. Now, the likelihood of getting a fit may be even greater."

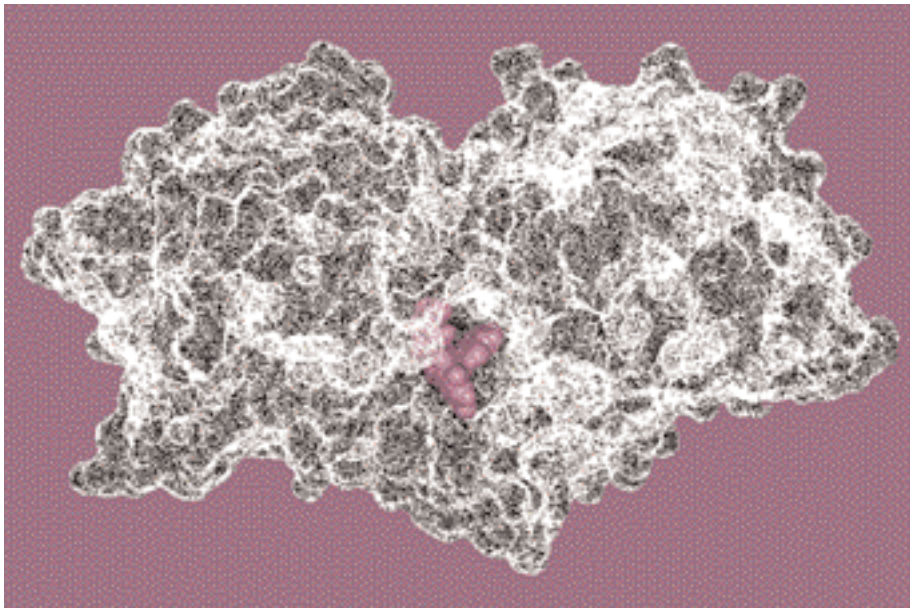
Into the Laboratory

Once possible ligands have been identified computationally, they must be tested in the laboratory to see whether binding actually occurs. Mass spectrometry (MS) and NMR spectroscopy are both effective for testing ligand–protein binding. NMR examines binding in the solution state, while MS looks at binding in the gas phase. MS typically requires much smaller samples, but it cannot handle certain compounds or chemical buffers. NMR can examine mixtures of compounds more easily and determine which combinations bind best in solution. Both techniques can identify where on the target protein binding is occurring.

The initial computational screening process to find new compounds that bind to tetanus neurotoxins resulted in 100 possible ligands that were predicted to bind to one of two sites (site 1 and site 2) on the tetanus neurotoxin's targeting domain. Experiments using electrospray ionization–mass spectrometry (ESI–MS) suggested that



(a) The x-ray crystal structure for the tetanus toxin showing how the amino acid chain is folded and (b) its calculated molecular surface showing sites 1 and 2, predicted binding sites for ligands.



The predicted structure of the tetanus–lavendustin A complex. Lavendustin A is shown in purple binding to site 2.

7 of the first 13 tested compounds bound to the toxin. With ESI–MS, ligand binding is confirmed when a new mass peak appears at the expected mass-to-charge ratio for the ligand–tetanus complex.

The antitumor drug doxorubicin was discovered to be the best fit at site 1. The binding of this ligand to site 1 was later confirmed by x-ray crystallography of doxorubicin–tetanus toxin and doxorubicin–botulinum toxin complexes. For site 2, the same MS method was used to screen 1 of 100 compounds, six of which were observed to bind. The figure above shows one of these ligands, lavendustin A, docked into site 2 in the predicted structure of the tetanus–lavendustin A complex.

The six ligands predicted to bind to site 2 were then screened for binding to the targeting domain using NMR. The six molecules were tested individually, as mixtures of different combinations of the compounds, and in the presence or absence of the known site 1 binder, doxorubicin.

When examined by NMR, small molecules exhibit weak, negative signals referred to as NOEs (nuclear Overhauser effects). Large molecules such as proteins exhibit strong, positive NOEs. When small molecules bind to proteins, the characteristics of the NOE for the large molecule are transferred to the small molecule. Thus, strong NOEs are detected for ligands that bind to the protein.

The NMR screening of mixtures containing the six predicted site 2 ligands confirmed that four bind to tetanus toxin in solution. Using a novel transfer NOE (trNOE) competition assay, researchers have determined that three of these ligands bind in the same site, presumably at site 2. The fourth ligand was determined to bind in a third site distinct from site 1 and site 2.

NMR experiments were also performed to evaluate how possible structural changes induced by the binding of one ligand in site 1 could influence the binding of the second ligand in another site. In these experiments, doxorubicin, which was added first, remained bound to site 1 throughout the additions of all six of the predicted site 2 ligands. The mixture containing doxorubicin and lavendustin A produced the strongest positive trNOE signal in the presence of the tetanus toxin. This experiment confirmed that both lavendustin A and doxorubicin bind simultaneously to the toxin, indicating that each must bind to a different site.

"Unfortunately, this assay cannot define the location of the binding site," says physical chemist Monique Cosman, leader of the NMR group at Livermore. "But since doxorubicin is known to bind to site 1, we know that lavendustin A must bind to a different site, which may be site 2.

By performing these trNOE binding experiments with pairs of molecules that were determined to compete for binding to the same site, Cosman developed a new NMR method for identifying the relative strength of binding of each ligand to a particular site on the protein. MP-biocytin, another molecule that binds to site 2, did so with a relatively lower affinity than lavendustin A. The affinity of the third ligand is similar to that of lavendustin A, but it was not studied further because it is too perishable.

Mass spectrometry was then used to verify where the molecules are binding. Chemist Sharon Shields developed a new method that combines MS with proteolysis, a process in which a protein is digested by enzymes. "This is unique," she notes. "Now we can study solution-phase biological processes using a gas-phase mass spectrometric method."

She first treated the targeting domain of tetanus toxin with proteases that make clips in the amino acid chain either alone or on the tetanus–doxorubicin complex using various ratios of doxorubicin to the neurotoxin. Then she used matrix-assisted laser

desorption ionization and ESI–MS to determine the pattern of enzymatic degradation that had occurred. In the tetanus–doxorubicin combinations, doxorubicin prevented the enzyme from digesting the protein at the binding site by limiting access to the amino acids located in that region.

The figure below shows a map of peptides (amino acid chains) produced by digesting the tetanus–doxorubicin complex compared to the tetanus toxin alone. In this experiment, Shields used the enzyme trypsin. The decreased abundance of peptides indicates the location where binding is occurring. That location contains amino acids 299–304, 351–376, and 394–434. Molecular docking calculations had predicted that doxorubicin would reside near amino acids 356, 358, 359, 407, 409, 419, 427, and 437. These predictions are a close match to MS results. Comparable locational experiments using other enzymes had similar results.

Shields also found that the presence of doxorubicin induces subtle changes in the tetanus toxin's three-dimensional structure, suggesting that the protein may envelope, or wrap around, doxorubicin when it binds. Further experiments are needed to confirm these results.

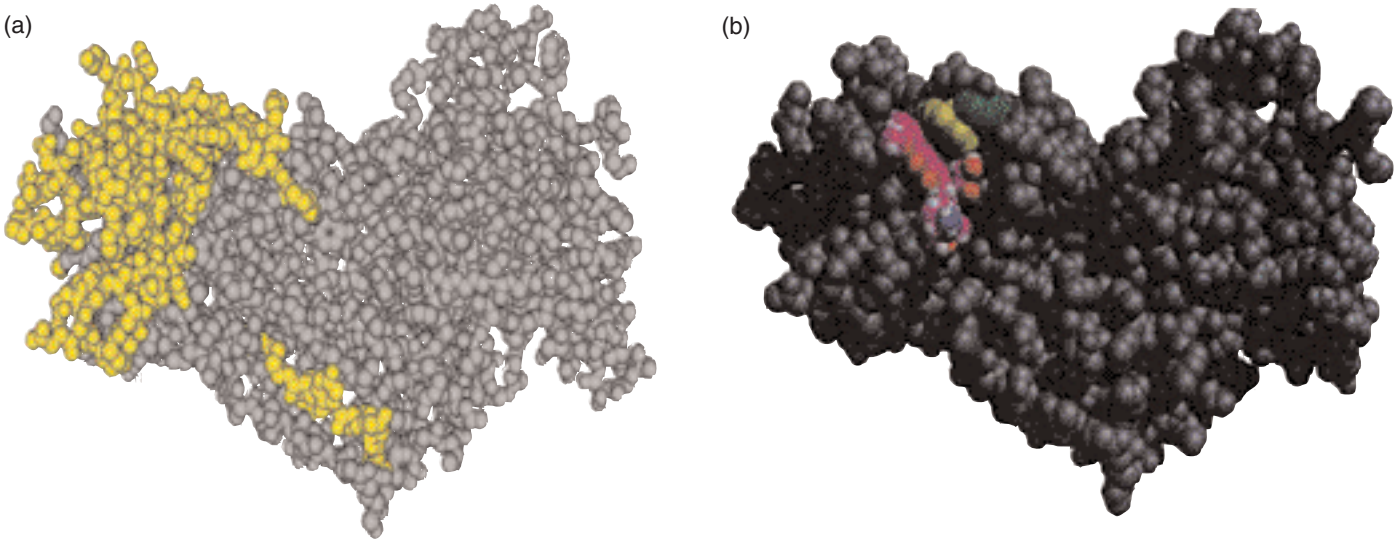
Creating a New Molecule

Synthetic chemist Julie Perkins has the job of linking the two molecules that bind to sites 1 and 2 to create a new HAL. This is the critical step. She is experimenting with linkers that will connect doxorubicin and MP-biocytin as well as doxorubicin and lavendustin A. "We know that each of these compounds binds individually to sites 1 and 2, but because they bind weakly, they can also float away," Perkins says. "When the compounds are linked together, they are much more likely to stay bound.

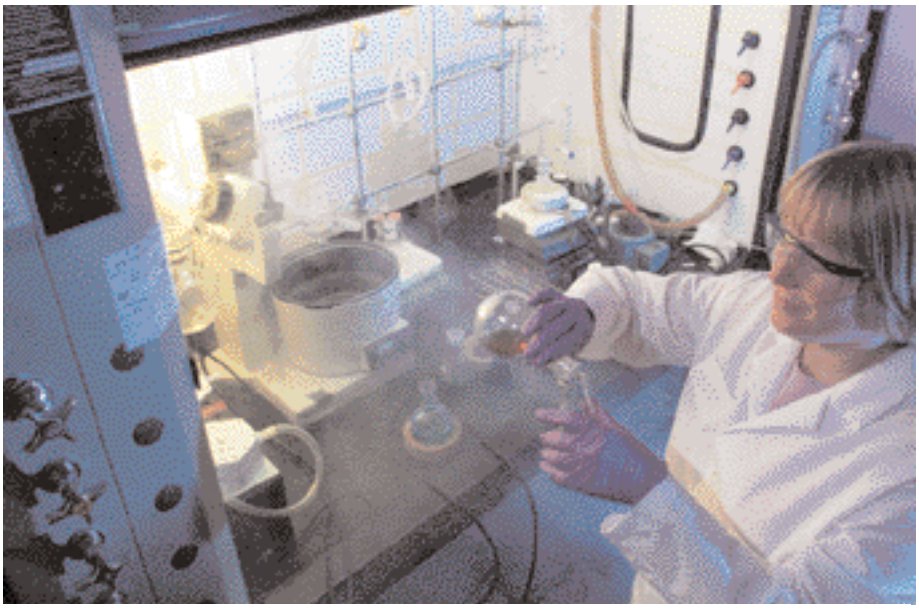
She is starting with the amino acid lysine as a linker. Lysine is an ideal building block because it has three distinct functional groups upon which she can perform synthetic chemistry experiments. Many derivatives of lysine are commercially available as well. The molecules that have been identified to bind into site 1 and site 2 can either be attached directly to lysine, resulting in their close proximity, or with a linker, which increases the distance between them. Increasing the distance between the two compounds with a flexible chain may also help increase the affinity of the ligand for the protein.

"To achieve maximum affinity of the ligand for the protein, we have to find the optimal length and rigidity of the linker," says Perkins, "and that can only be done experimentally." She is experimenting with a flexible glycol chain that can be attached to the lysine to increase the distance separating the two ligands.

Once she has synthesized each new compound containing the two linked ligands, conventional binding studies will identify the highest affinity and most selective ligand combinations.



(a) This map of peptides (amino acid chains) compares the doxorubicin–tetanus toxin complex to the tetanus toxin alone. Amino acids in yellow represent the peptides that showed a decreased abundance, which indicates that binding is occurring. (b) Computational docking studies predicted that binding would occur at the location shown. The two match quite well.



Synthetic chemist Julie Perkins works to link two molecules, each of which binds to two protein binding sites. The new molecule will bind more strongly and securely to a specific toxin protein than the individual molecules can.

These studies will determine how tightly the HALs bind and confirm that they selectively bind only to *Clostridium* neurotoxins.

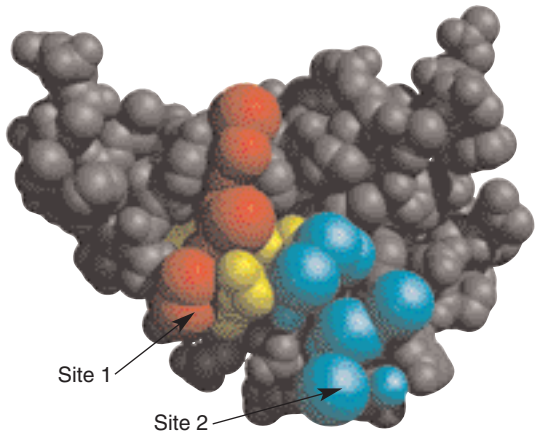
Targeting Cancer

For cancer therapy, the challenge is to synthesize molecules that bind with high affinity to each cancer cell without themselves generating an immune reaction from the body. Targeting molecules therefore must be smaller and more specific and have higher affinities than natural antibodies. They should also not be made of proteins, which elicit an immune response from the body.

The goal is to use these small, exceptionally high-affinity molecules to deliver a lethal radiation dose directly to a tumor. In this case, the HALs would be tagged with radioactive isotopes and introduced into the body. Research all over the world is focused on this new technique, known as isotopically enhanced molecular targeting.

To create new HALs for cancer treatment, Livermore is using the same process developed for producing HALs that bind to toxins and pathogens. The first project will be a HAL for a receptor protein found on the surface of non-Hodgkin's lymphoma, HLA-DR10. The crystal structures of four HLA-DR molecules are known, and unique binding sites on the HLA-DR10 protein have been identified using computer models of the protein generated by computational biochemists Adam Zemla and Daniel Barsky. Computational docking experiments are under way.

The HAL developed for binding HLA-DR10 and targeting human lymphomas will be designed to rapidly pass through the liver and kidney and thus minimize the systemic damage that can occur when antibodies carry radionuclides. "We are striving to convert the meaning of the word 'cancer' from 'fear, pain, suffering, and death' to 'just another treatable disease,'" says Balhorn.



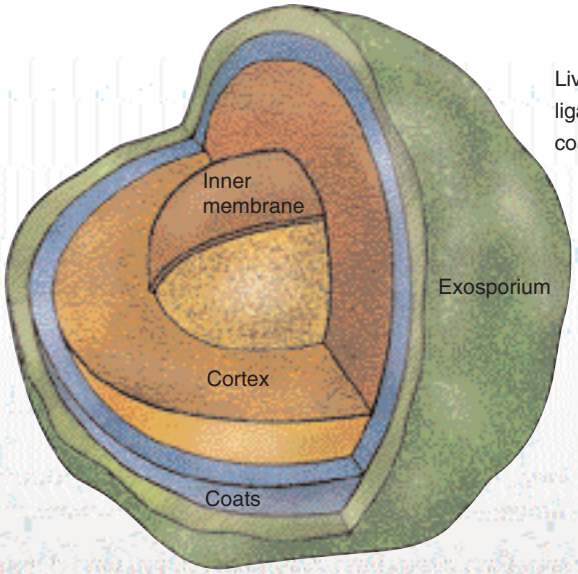
Sites 1 and 2 on the HLA-DR10 molecule (a protein receptor for non-Hodgkin's lymphoma) have been identified.

Targets of Unknown Structure

When a target protein's structure is not known, the team will use a different route to design and synthesize HALs. Computers cannot be used to predict the binding of molecules to sites on these proteins. But NMR and MS processes that are being developed and fine-tuned now for identifying ligands that bind to known protein structures will identify ligands that bind to

unknown structures.

Libraries of molecules will be experimentally screened for their ability to bind to the protein using a combination of Cosman's NMR technique and mass spectrometry methods being developed by chemist Lori Zeller. The molecules that bind will be segregated into sets that bind to different sites. Perkins will then synthesize all possible combinations of pairs of these small molecules using a series of different-size linkers. With Livermore's new Fourier transform ion cyclotron resonance mass spectrometer, mixtures of the HALs and protein can be quickly screened to identify the particular combination of ligands and linkers that produce HALs that bind to the protein. This approach should work well for creating detection reagents for pathogens. In collaboration with groups at Porton Down Defense Science and Technology Laboratory in England, Livermore researchers will design the first HAL for a protein with an unknown structure to bind to a protein on the coat of the anthrax spore.



Livermore will design a high-affinity ligand to bind to protein in the spore coat of *Bacillus anthracis* (anthrax).

Measuring Success

The Livermore team will soon produce its first HAL for the *Clostridium* neurotoxins. To know whether this work has been successful—whether the ligand works as designed in a bioagent detector—the team will send its results to the Department of Defense's Critical Reagent Program to be assessed for quality and specificity.

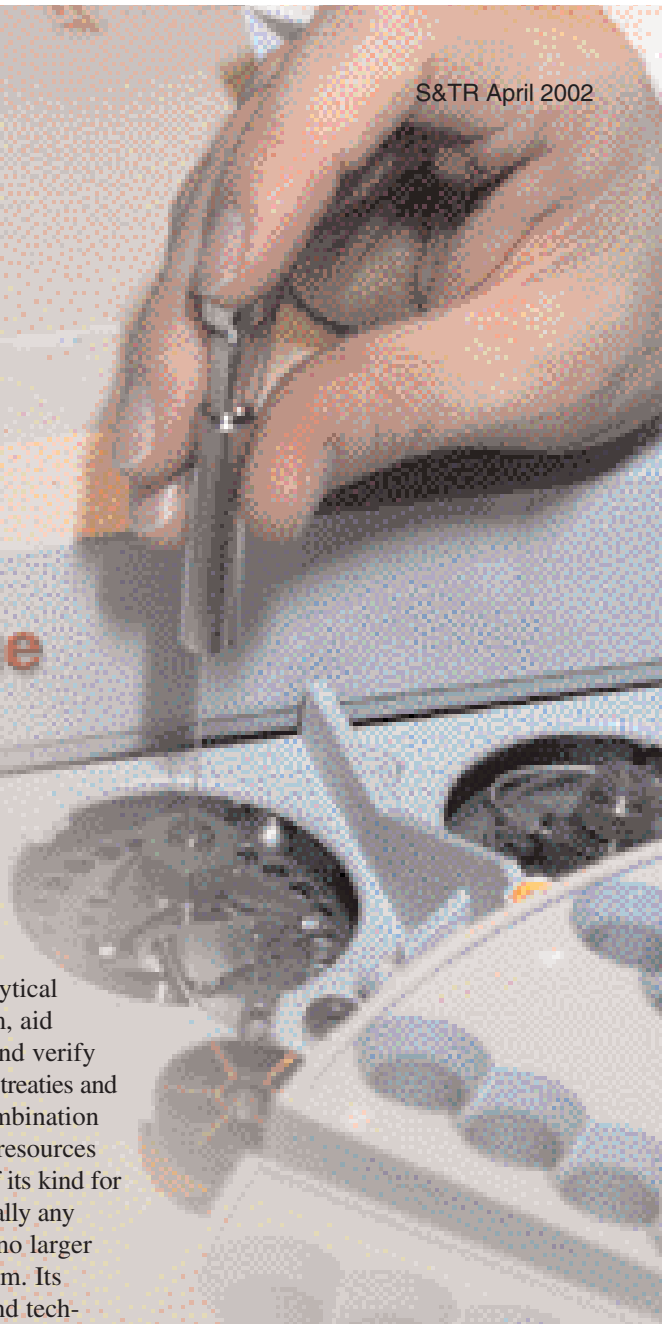
In the war against bioterrorism, the best defense begins with having the best possible data. Work has begun on docking studies to identify binding sites on the light chain of botulinum toxin. In this case, the goal is to synthesize HALs that can distinguish between the different types of *Clostridium* neurotoxins. That kind of fine-tuning is essential for accurate bioagent detection and identification during a crisis.

—Katie Walter

Key Words: antibodies, bioterrorism, botulinum toxin, cancer treatment, *Clostridium* neurotoxins, high-affinity ligands (HALs), mass spectrometry (MS), nuclear magnetic resonance (NMR), protein structure, synthetic chemistry, tetanus.

For further information contact Rod Balhorn (925) 422-6284 (balhorn2@llnl.gov).

Forensic Science Center Maximizes the Tiniest Clue



Livermore chemists are coaxing a wealth of information from increasingly small samples.

WHILE Lawrence Livermore's national security accomplishments have received much publicity over the years, one Laboratory organization has gained such a stellar reputation among law enforcement, intelligence, and emergency response agencies that it is cited by Tom Clancy in his novel *Shadow Watch* (Berkley Books, 1999):

"I've requested assistance from the Forensic Science Center in San Francisco. It's at the Lawrence Livermore National Laboratory. I don't know if you're familiar with them."

"They did evidence analysis on the Unabomber case, the Times Square and WTC bombings in New York, probably hundreds of other investigations," Nimec said. "Uplink's had a relationship with them for years, and I've worked with them personally. The LLNL's the best group of crime detection and national security experts in the business."

Founded in 1991, the Laboratory's Forensic Science Center (FSC) offers a comprehensive range of analytical expertise to counter terrorism, aid domestic law enforcement, and verify compliance with international treaties and agreements. The center's combination of human and technological resources has made it among the best of its kind for collecting and analyzing virtually any kind of evidence, some of it no larger than a few billionths of a gram. Its resources, expertise, tools, and techniques are applied to all kinds of cases, from the September 11 World Trade Center attack to the spread of anthrax spores, from multiple homicides to nuclear materials smuggling.

FSC has a staff of 15 personnel, mostly chemists, with expertise in analytical chemistry, organic chemistry, inorganic chemistry, nuclear chemistry, toxicology, pharmacology, special coatings, and forensic instrument design and fabrication. The center also draws upon the resources of experts in Livermore's Chemistry and Materials Science and Nonproliferation, Arms Control, and International Security directorates.

The center's approach to forensic analysis maximizes the information that can be obtained from sometimes extremely small samples of explosives residue, dust particles, hair strands, blood stains, radioactive isotopes, drugs, chemicals, and clothing fibers. As Brian Andresen, until recently FSC director, says, "We're probing the lower limits of detection for many types of compounds isolated during an investigation." Even the tiniest quantities, says Andresen, are usually enough to provide compelling evidence that holds up in court. The minuscule amounts of oils remaining on fingerprints, for example,

can tell the general age of suspects, their diet, and whether they smoke. In that respect, says Andresen, "Everything someone does leaves a chemical or biological signature that we can investigate."

Many forensic research projects have required FSC personnel to develop new analytical tools, forensic techniques for analyzing trace amounts of evidence, and unique sampling procedures. Several new, portable instruments have been developed that are capable of detailed analysis in the field. These tools provide important advantages when dealing with substances that may be unstable, perishable, or too toxic to bring back to the Laboratory.

Supporting International Security

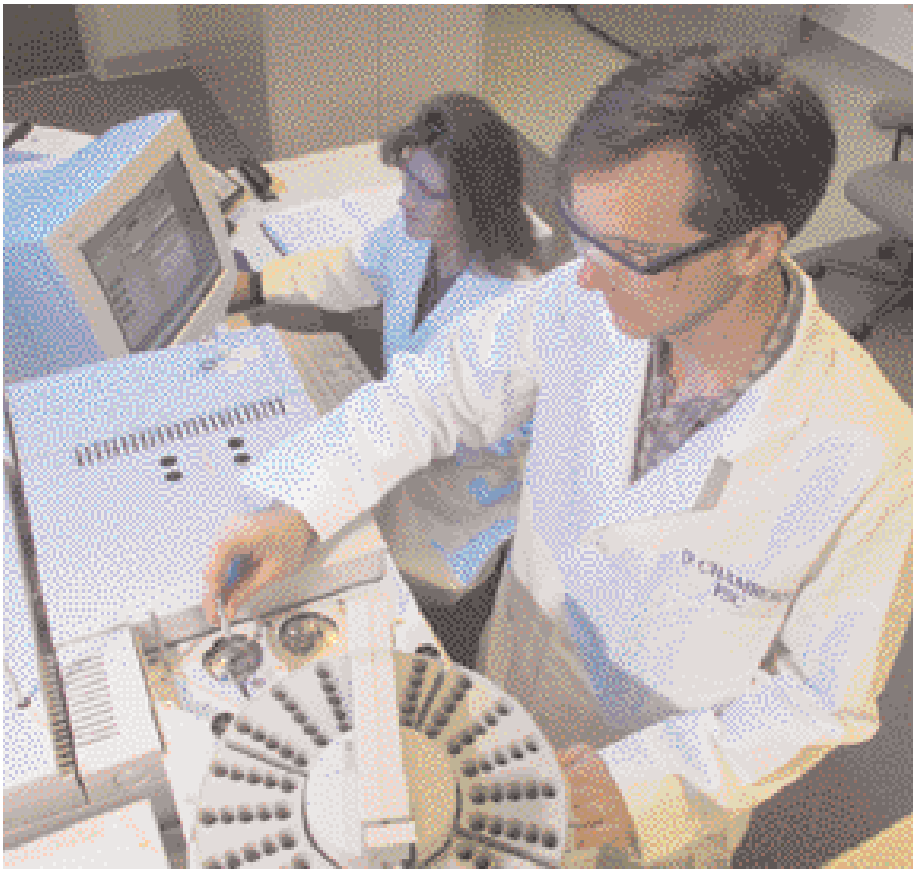
Andresen notes that the term "forensic science" used to apply only to the scientific analysis of evidence for civil or criminal law. Increasingly, however, forensic analyses done at FSC are broadening that definition to include support for monitoring or verifying compliance with international treaties and agreements, particularly those involving weapons of mass destruction, and for countering threats of terrorism. For example, the center is contributing to the National Nuclear Security Administration's (NNSA's) Chemical and Biological National Security Program to develop and field advanced technologies to better prepare for, detect, and respond to chemical or biological incidents in the U.S.

In light of its demonstrated capabilities to analyze minute specimens, FSC was selected by the U.S. State Department in 2000 to support the Organization for the Prohibition of Chemical Weapons (OPCW) as the second U.S. certification laboratory. (The other facility is the Edgewood Chemical and Biological Analytical Center in Maryland.)

OPCW, based in the Netherlands, is responsible for implementing the Chemical Weapons Convention, which bans the production, stockpiling, or use of such weapons as nerve agents and blister agents. OPCW-designated laboratories test samples collected by OPCW inspectors from sources around the world to determine whether the samples contain chemical weapon agents, their precursor chemicals, or decomposition products. The convention stipulates that all samples must be analyzed at the two OPCW-designated laboratories. Federal legislation requires that all samples taken from a U.S. facility be tested in a U.S. laboratory that is OPCW-certified.

FSC has established a separate chemical weapons analysis laboratory that is certified by the American Association for Laboratory Accreditation. To date, no actual samples have been officially collected from any site or analyzed at any laboratory. FSC, however, has been required to analyze and identify constituents of mock samples supplied by the OPCW as part of a series of proficiency tests.

According to FSC's Armando Alcaraz, "Passing the tests is a very challenging task because the samples might contain literally thousands of chemicals that are linked to chemical weapons manufacturing." He notes that the samples are sometimes spiked with certain materials to deliberately try to



Heather King and David Chambers demonstrate Livermore's solid-phase microextraction (SPME) sampling technique for identifying and quantifying the chemical composition of physical evidence.

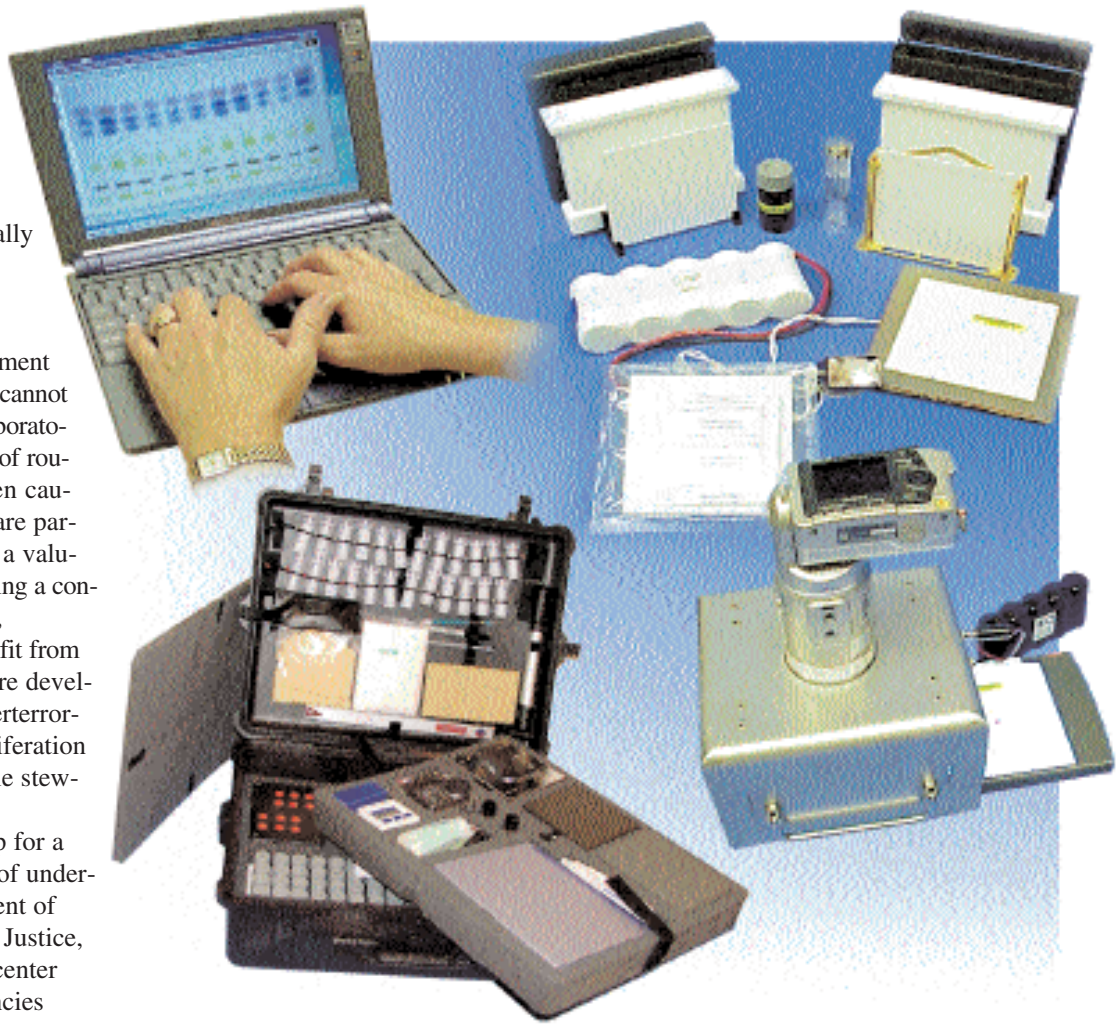
throw the analysis teams off track. Like the test samples, the real samples will be extremely dilute (that is, parts-per-million level) so that they can be shipped commercially or sent through the mail.

Helping Law Enforcement

FSC also assists law enforcement agencies with special needs that cannot be handled by standard crime laboratories. "We're not in the business of routine police lab work," Andresen cautions. However, for cases that are particularly difficult, FSC may be a valuable resource capable of providing a conclusive analysis. In this respect, law enforcement agencies benefit from Livermore technologies that were developed initially to support counterterrorism efforts, detect nuclear proliferation activities, and advance stockpile stewardship.

Under the 1998 "Partnership for a Safer America" memorandum of understanding between the Department of Energy and the departments of Justice, Commerce, and Treasury, the center provides law enforcement agencies such as the Federal Bureau of Investigation (FBI), the U.S. Customs Service, and the Bureau of Alcohol, Tobacco, and Firearms with new crime-fighting technologies. This agreement provides a framework for formal working relationships to facilitate the transfer of DOE technology and technical expertise to law enforcement.

FSC deputy director Pat Grant notes that supporting law enforcement increases the center's expertise and shortens the turnaround times for sample analysis. "Anytime we analyze questioned samples important to a real-world investigation, we are honing our skills. It's a much more interesting and stimulating experience than participating in an exercise."



Scientists from the Forensic Science Center have miniaturized thin-layer chromatography to make it suitable for field use. The portable system includes appropriate reagents, glass plates, a digital camera, and a notebook computer.

Shrinking Instruments

Some of the center's most enduring accomplishments are new tools it has developed for intelligence, law enforcement, and health professionals working in the field. These compact, battery-powered tools provide mobile chemistry laboratories. Because they eliminate the need to ship samples back to a standard laboratory for analysis, the portable technologies greatly speed decision making.

For example, FSC scientists have miniaturized and modernized thin-layer chromatography (TLC), a well-established laboratory procedure that identifies compounds belonging to the same general chemical class. FSC chemists made TLC technology suitable for field use with a portable system that fits inside a suitcase and weighs about 23 kilograms. Although the portable system uses minimal equipment and chemical reagents, it is highly specific and sensitive. The kits can be used to analyze two sets of

samples simultaneously, with each set containing about 10 samples. Depending on the compounds being analyzed for, the entire process takes 10 to 20 minutes to complete.

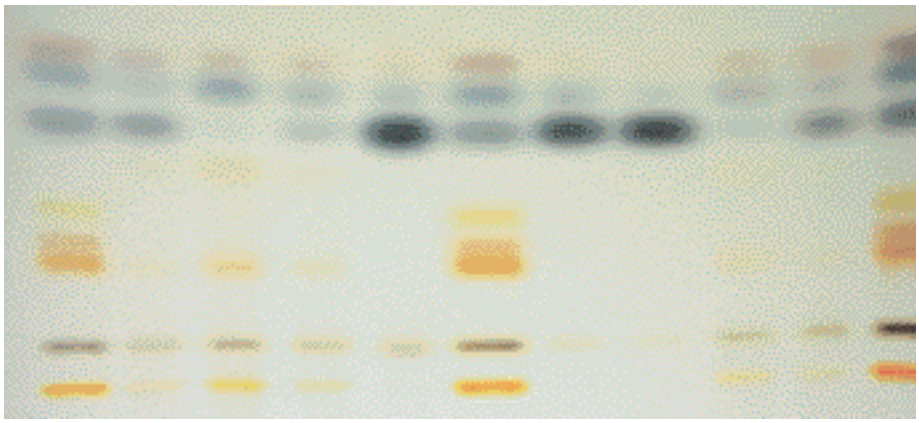
TLC works by separating compounds over the distance they move up a glass plate. Tiny amounts of samples are placed just above the bottom edge of a TLC plate, the plate is placed in a small solvent reservoir, and the solvent moves up the plate by capillary action. A commercial digital camera captures the resulting patterns of dark spots that develop, which are analyzed on a notebook computer using a software program originally developed for the analysis of DNA. Based on the distance the samples have traveled, together with their color and intensity, the computer program identifies the compounds and their relative concentrations.

The center's portable TLC kits are tailored to detect chemicals indicative of chemical weapons, high explosives, propellant stabilizers, or illegal drugs. Each specialized kit includes solvents and developing reagents that are specific to the compounds of interest.

The TLC system was originally developed for the U.S. Army to quickly detect propellant instabilities within the nation's munition storage depots. Propellants (especially high explosives) require stabilizers to prevent them from spontaneously igniting. Because stabilizers are depleted by long exposure to environmental conditions, the Army needed a way to quickly determine the safety of large numbers of munitions. The center's TLC system requires only 50-milligram samples of explosive, instead of the gram quantities typically required by other methods, and 15 minutes for each group of 20 samples, allowing many more samples to be analyzed and at much lower cost than is possible using traditional methods. "Army personnel without a degree or extensive training in chemistry can do this work," says FSC chemist Jeff Haas.

Over a few days in 1998, the portable system successfully characterized the contents of more than 1,200 unearthed mortar rounds discovered in a shallow excavation site at an Army base in Massachusetts. (See *S&TR*, December 1998, pp. 21–23.) The system is now deployed at several other Army facilities as well as by National Guard units.

The system is also used in instances where analysis speed is essential. In light of repeated success by a variety of users, the center is transferring the portable TLC technology to private industry for commercialization and widespread availability to federal and state law enforcement, customs, and environmental agencies.



The portable thin-layer chromatography (TLC) system separates compounds as they move up a glass plate placed in a small solvent reservoir. Based on the distance the compounds travel, together with their color and intensity, a computer identifies the compounds and their relative concentrations. Above is a typical TLC analysis done to detect propellant instabilities by measuring the amount of stabilizer compounds.

Advanced Tools for Field Use

While TLC is effective for identifying classes of chemicals that are specifically targeted, the task of completely characterizing samples in the field requires a more sophisticated instrument such as the gas chromatograph–mass spectrometer (GC–MS). An essential tool in every major analytical laboratory, a GC–MS can detect ultratrace quantities of organic compounds weighing a billionth of a gram or less. The gas chromatograph first slowly heats a sample to about 250°C. As the sample's volatile constituents travel down a long capillary column, they separate according to their vapor pressures and chemical affinities. As they flow into the mass spectrometer, the compounds are bombarded with an electron beam that fragments molecules into ions that constitute a unique fingerprint of that compound for positive identification.

FSC staff scientists have shrunk the standard 114-kilogram laboratory GC–MS to about 28 kilograms; it now fits inside a wheeled suitcase. The self-contained portable device, comparable in sensitivity and selectivity to a standard unit, contains a power generator, vacuum pumps, and laptop computer. The result is an instrument that significantly improves on-scene investigation and evidence collection.

Because of its ability to analyze samples to parts-per-billion sensitivity

within 15 minutes, this portable GC–MS can be used to support nonproliferation activities, incident response, and law enforcement investigations. For example, the instrument can precisely identify compounds indicative of the manufacture of chemical warfare agents and illicit drugs. The instrument is currently being manufactured under license to industry.

Identification with Lasers

Although many tools used by FSC personnel depend on analyzing tiny amounts of chemicals that are found in a vapor phase above a liquid or some solid materials, most solid objects, such as human hair or clothing, do not have a significant vapor pressure and thus do not lend themselves easily to GC–MS analysis. However, center personnel can vaporize these solid samples with an extremely fine laser beam to generate wisps of product that contain identifying compounds.

The technology is called imaging laser-ablation mass spectroscopy. The process combines a laser for vaporizing extremely small amounts of material, an ion trap and time-of-flight mass spectrometer for analysis, and a high-powered microscope for viewing. In this way, forensic scientists can collect and rapidly identify suspect chemicals.

The process can be used on almost any solid material—dirt, pieces of glass, paint chips, clothing fibers, strands of hair. The samples are placed inside an ion trap mass spectrometer, irradiated with a laser, and identified within a few minutes by the mass spectrometer. The process allows an investigator to "walk down" a hair shaft by drilling consecutive holes on the same hair with the laser and analyzing each volatile sample. "Because hair grows at a standard rate, the results can reveal a history of drug use or exposure to compounds used in biological or chemical weapons manufacturing," says FSC chemist Greg Klunder. He points out that the method could also be applied to samples of clothing or soil sticking to the shoes of someone suspected of developing chemical weapons.

A similar instrument still under development is capable of detecting chemicals in air and is well suited for high-speed aircraft sampling of exhaust smoke from chemical facilities. Potential applications include identifying hazardous spills, monitoring industrial stacks for certain compounds, and surveying the environment from a remote location
to detect chemical releases from a suspect facility.

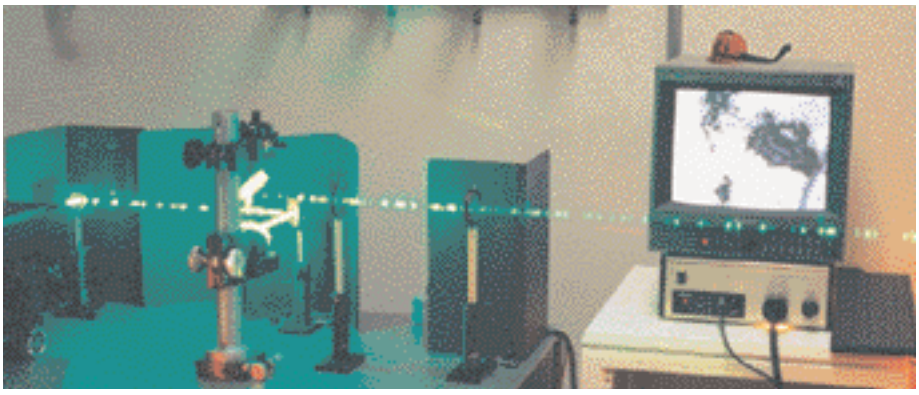
Wands of Collection

One of the center's most important developments has been the solid-phase microextraction (SPME) collection kits that use optical fibers as "chemical dipsticks" for safe and efficient sampling. "The technique has revolutionized the collection of forensic samples in the field," says FSC chemist Pete Nunes.

The technology uses commercial hair-size (100-micrometer-thick) fibers to capture organic vapors. The fiber, residing inside a syringe, is coated with a chemical polymer that, when exposed to the ambient environment for a suitable amount of time, can collect thousands of different compounds by acting as a chemical sponge. The polymer coatings are specific for different types of compounds such as chemical warfare agents, high explosives, or illegal drugs.

The collection technique requires no solvents, sample workup, or additional equipment typically associated with obtaining evidence. The fibers can be inserted directly into a portable or stationary GC–MS for immediate analysis.

Forensic Science Center chemist Del Eckels uses the 28-kilogram portable gas chromatograph–mass spectrometer that fits inside a wheeled suitcase. The portable unit, comparable in sensitivity and selectivity to much larger and heavier units, permits fast on-the-scene chemical analysis.



The imaging laser-ablation mass spectrometer combines a laser for vaporizing extremely small amounts of material, an ion trap time-of-flight mass spectrometer for analysis, and a high-powered microscope for viewing.

Nunes says that because the fibers are fragile, they had never been taken into the field. To overcome their fragility, an FSC team developed rugged aluminum transport tubes, with each tube securing one syringe and fiber. A group of five tubes is contained in each kit. The hermetically sealed tubes prevent any possibility of cross-contamination and support chain-of-custody requirements. A sampling port in the bottom of the tube permits assaying the contents in a glove box before the tube is actually opened.

SPME sampling is being put to good use by FSC weapons scientist David Chambers to monitor nuclear weapon warheads safely. This activity is part of the NNSA's Stockpile Stewardship Program to maintain the safety and reliability of the nation's nuclear stockpile.

Center Plays Role in Famous Law Enforcement Cases

The Forensic Science Center (FSC) has played a pivotal role in several well-publicized criminal investigations. For example, FSC examined the composition and structure of tiny bomb fragments containing trace metal and chemical residues in the Unabomber case.

The center provided analysis and testimony leading to the conviction of Fremont, California, bomber Rodney Blach, a former Chicago Police Department forensic investigator. Blach was convicted of planting bombs during 1998 at the homes of the police chief, a city council member, and others. Former FSC Director Brian Andresen helped investigators from the federal Bureau of Alcohol, Tobacco and Firearms (ATF) to reconstruct what Tom Rogers, assistant district attorney, characterized as "the largest as well as the most electronically sophisticated domestic pipe bombs the ATF had ever encountered." Rogers said, "The electronic aspects of the devices were beyond the expertise of anyone at the ATF."

FSC supported the Democratic National Convention in 2000 by providing a mobile forensic laboratory for the Los Angeles County Sheriff's Terrorist Early Warning Group. The center was also instrumental in interpreting factors surrounding the death of Gloria Ramirez, who made several hospital emergency room personnel violently ill in a well-publicized Southern California case.

FSC helped prosecutors in Glendale, California, rearrest Efren Saldivar, the self-proclaimed Angel of Death and alleged killer of many terminally ill hospital patients. FSC scientists performed toxicology analyses on exhumed tissues from 20 patients. They didn't expect to find anything. However, with the help of completely new techniques, including sample collection procedures developed by the center, they were able to identify the drug Pavulon in the bodies of six of the deceased patients. The rearrest of Saldivar was based primarily on the center's findings

Identifying Bullet Fragments

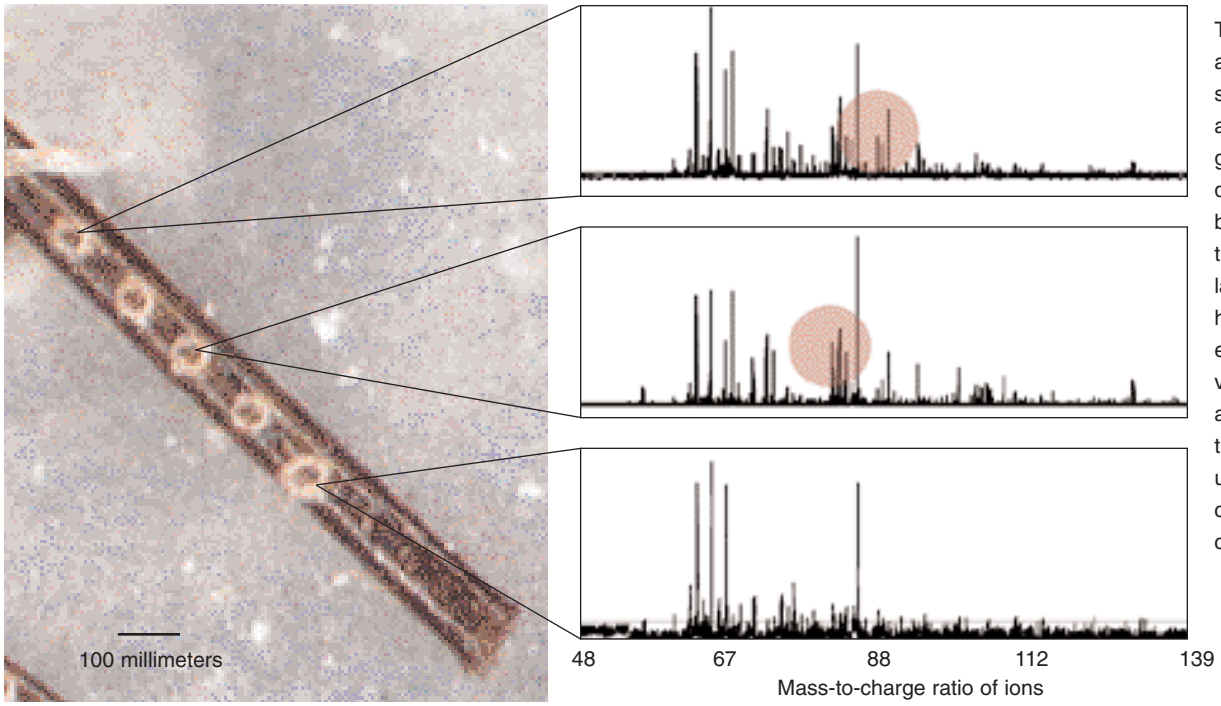
FSC came to the aid of Kings County, California, authorities who were stymied by an execution-style triple homicide. The evidence included a variety of bullet fragments but no weapons. Investigators found corroded, expended casings scattered around the grounds where the suspects lived. FSC personnel led by Rick Randich chemically treated the casings to remove corrosion and then used optical and scanning electron microscopes to match the crime-scene evidence with residence specimens. The staff published its restoration methods as an aid to other agencies.

The center analyzed debris from an explosion that killed a scientist during a 1992 cold fusion experiment at SRI International in Palo Alto, California. In testing the explosion debris, FSC chemists discovered a trace amount of oil in the interior of the SRI electrochemical cell. They determined that a likely source of this oil was lubricating fluid that remained from machining the metal cell components. They concluded that the high-pressure oxygen atmosphere of the electrochemical cell possibly created the potential for an explosive reaction with the oil.

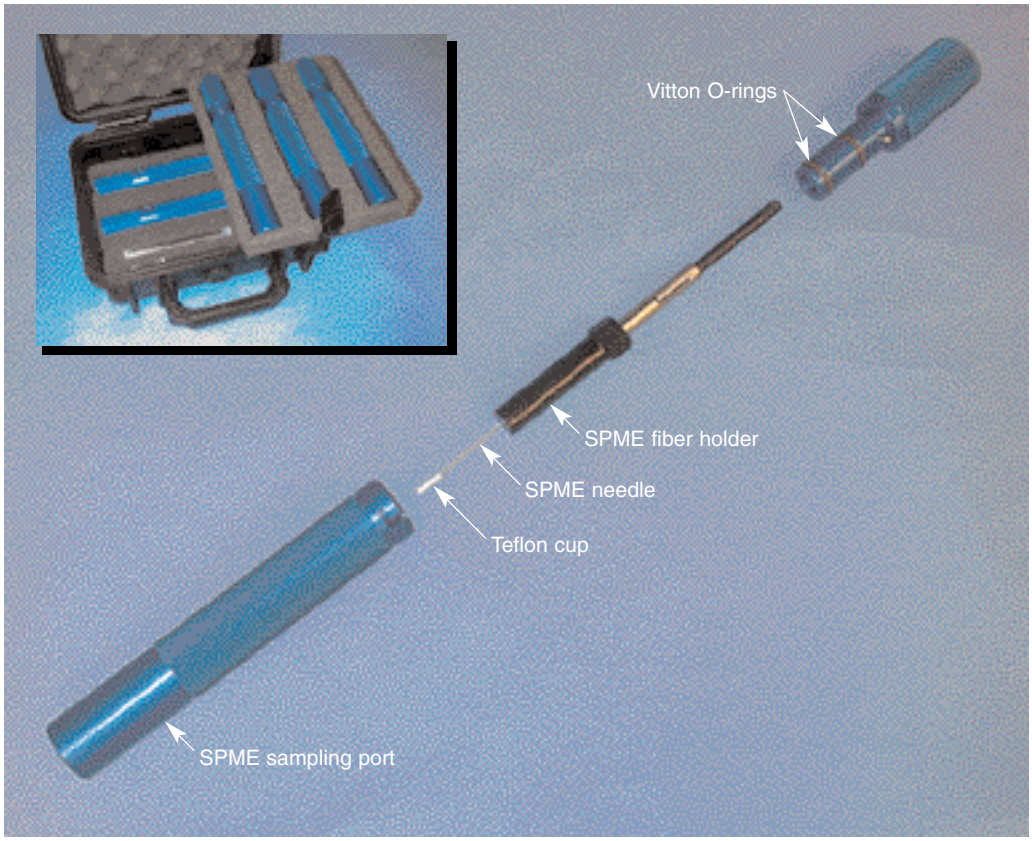
Many FSC investigations involve identifying unknown substances. One specimen brought to the center was a suspicious green liquid uncovered by the Federal Bureau of Investigation (FBI) during a search of a stolen cache of weapons. The container of the liquid was labeled "poison" and gave a dilution formula for use. FSC chemists analyzed the solution for chemical warfare agents but finally identified it as a concentrated cleaning agent.

Another extraordinary analysis centered on a shipment of white crystals in ampoules from China that was thought to be heroin. The powder was interdicted by the U.S. Customs Service and subsequently investigated by the FBI. FSC analyses identified the material as tetrodotoxin, a deadly marine neurotoxin derived from puffer fish. "The definite identification of tetrodotoxin was a real success story for the center," says Andresen.

In the past several months, FSC has been helping authorities to identify samples of substances suspected of being anthrax. Several of the specimens brought to the center by law enforcement officials were from the local community, while others were from locations at the Laboratory. None was found to be real anthrax; instead, the powders were determined to be food materials, dust, dirt, cell culture medium, and powdered paper.



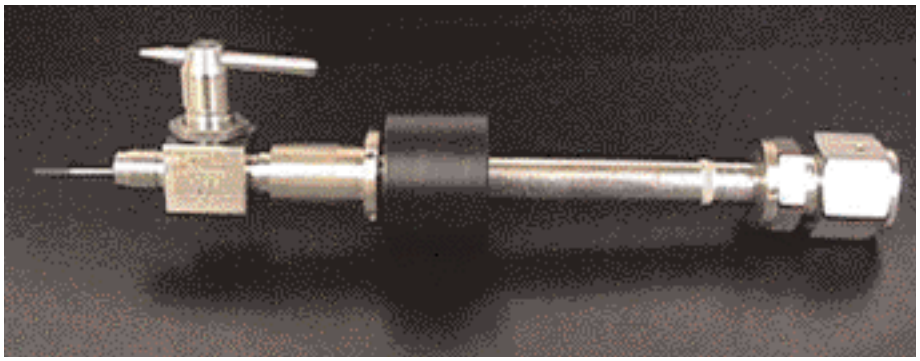
The imaging laser-ablation mass spectrometer allows an investigator to "walk down" a hair shaft by drilling consecutive holes with the laser on the same hair and analyzing each to obtain a volatile sample for a history of activities such as drug use or exposure to chemical weapon compounds.



The Forensic Science Center's solid-phase microextraction (SPME) collection kits use optical fibers as "chemical dipsticks" and (inset) rugged aluminum transport tubes for safe and efficient sampling. The technique has revolutionized collecting evidence in the field.

Chambers uses SPME's coated fibers to collect volatile and semivolatile molecules that are formed or outgassed from the nuclear and thermal breakdown of organic polymers and high explosives. Signs of outgassing can indicate problems such as corroded metal parts that need to be replaced. By monitoring for the presence of these chemical vapors, scientists are alerted to problems that may be developing inside the weapon.

The center has provided the FBI and other agencies with SPME field kits for the safe and rapid collection of chemical warfare agents. The kits are equally well suited for drug detection and arson investigations. FSC has also developed a new SPME transport tube that is smaller and lighter so that it can fit inside a shirt pocket. Both versions are being licensed to industry for sale to government agencies.



This solid-phase microextraction device is used to collect molecules that are formed or outgassed from the nuclear and thermal breakdown of organic polymers and high explosives contained in nuclear warheads. Signs of outgassing can indicate problems with parts that need to be replaced.

Always On Call

Although the Forensic Science Center was highlighted in a Tom Clancy novel, it is not fiction. It is a rich resource for the national security and intelligence communities and has proved itself a valuable ally to federal and state agencies alike. Just as they have for the past 10 years, FSC personnel will be on call for the next case and the next sample.

—Arnie Heller

Key Words: anthrax, Chemical and Biological National Security Program, Forensic Science Center (FSC), gas chromatograph–mass spectrometer (GC–MS), laser-ablation mass spectroscopy, Organization for the Prohibition of Chemical Weapons (OPCW), solid-phase microextraction (SPME), stockpile stewardship, thin-layer chromatography (TLC).

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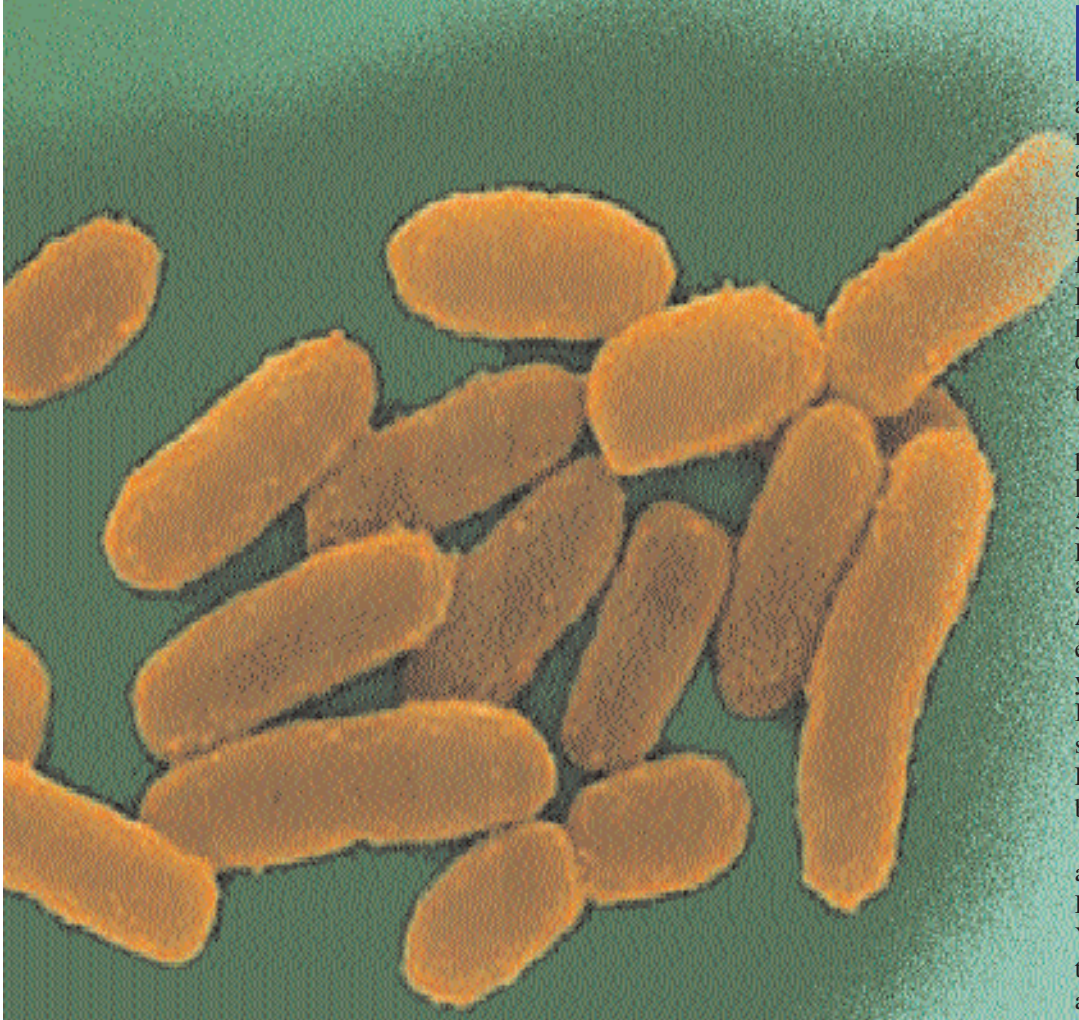
Tracking Down Virulence in Plague

How do the plague pathogen and its host interact? Scientists will apply the answer to understanding a larger set of possible agents of biological terrorism.



PLAGUE is potentially a deadly agent of bioterrorism. Unlike anthrax, which has been so much in the news lately, plague is highly infectious and can be readily passed from one person to another. The bite of a plague-infected flea or the inhalation of just a few cells of plague bacterium can kill. Like smallpox, plague can spread and kill large numbers of people very quickly. Fortunately, it can usually be treated with antibiotics.

History tells us how devastating a plague epidemic can be. In what is known as the Justinian epidemic, from 540 to 590 AD, plague spread from Lower Egypt to Alexandria to Palestine and on to the Middle East and Asia. At its peak, 10,000 deaths occurred every day in Byzantium. Eight hundred years later, in 1347, plague came to Italy from Asia or Africa, probably by ship. By 1351, fully one-third of Europe's population had died from bubonic plague.

This European epidemic is known as the Black Death or the Great Pestilence. In 1894, when Andre Yersin identified the tiny bacterium that causes plague, he named it *pestis* after the Great Pestilence. He tried to

name the genus *Pasteurella* after his mentor, Louis Pasteur. But *Yersinia*, after its discoverer, is the name that stuck.

Today, *Yersinia pestis* is one of several infectious diseases and agents of bioterrorism that researchers across the Department of Energy complex are studying as part of the Chemical and Biological National Security Program. This program comes under the purview of DOE's National Nuclear Security Administration (NNSA). At Livermore, the work on *Y. pestis* also receives support from Laboratory Directed Research and Development.

Scientists at Livermore have developed DNA signatures for *Y. pestis* that can be used to quickly detect and identify plague outbreaks. (See "Uncovering Bioterrorism," *S&TR*, May 2000, pp. 4–12.) Signatures for nine strains of the disease have been submitted to the Centers for Disease Control and Prevention in Atlanta, Georgia, where they are undergoing a rigorous validation process.

Livermore's DNA-based detection method proved its mettle in northern Arizona last June when it was used to identify a plague outbreak in prairie dogs in just four hours. Standard detection processes, which require growing the suspected bacteria in a laboratory, take 36 to 48 hours.

For a plague detector to be truly effective, it must do more than simply indicate the presence of a specific organism known to cause plague, says Pat Fitch, who leads Livermore's Chemical and Biological National Security Program. The detector also must be able to identify the specific traits found in atypical plague-causing organisms. Scientists know of several hundred strains (or isolates) of *Y. pestis*, and they do not all behave in precisely the same way. A few strains are believed to have been genetically modified or engineered to be more deadly. There have also been two clinical cases of naturally occurring antibiotic-resistant plague. Knowing the precise identity of a strain of plague—or of any infectious disease, for that matter—could help physicians treat a patient properly.

Plague research at Livermore currently is focusing on what makes *Y. pestis* so virulent and able to overcome the defenses of a host organism. Fitch is leading the Pathogen Pathway Project, using plague as a prototype for the functional genomics of a larger set of pathogenic agents that could be used in biological terrorism. (See the box on p. 6 for more information on functional genomics.)

Besides building better detectors, work on the *Y. pestis* genome will also lead to a better understanding of pathogenicity and better vaccines and treatments for the disease. The ultimate goal, says Fitch, "is to produce a computer model that simulates the workings of a cell so that we can better manage exposure to pathogens."

Plague as Prototype

The highly contagious *Y. pestis* is an excellent model for studying the interactions of a pathogen and its host. In the case of *Y. pestis*, the host may be a flea, a rodent, or a human. Fleas carry plague bacteria and help transmit the disease. Once an infected flea bites a rodent or human, the bacteria begin to multiply in the new host, and their virulence shifts into high gear. *Y. pestis* circumvents the host's defenses by injecting into host cells a series of virulence factors that inhibit the response of the immune system.

Earlier research has shown that when *Y. pestis* is grown at the body temperature of a flea (26°C), its cells divide, but it does not express (turn on) many of the genes that make it virulent in rodents and humans. When the tem-

(a) *Yersinia pestis*, which causes plague, is a pathogen likely to be used by terrorists. (b) Its DNA forms loops, unlike human DNA, which forms strands. Scientists studying it screen between two to five million of its nucleic acid bases to find unique regions (circled). Using polymerase chain reaction technology, the unique regions can be amplified thousands of times and processed to identify and characterize *Y. pestis*.

perature increases to 37°C (human or rodent body temperature), the bacterium begins to produce the proteins essential to its virulence. This virulence mechanism can be induced in the laboratory, making plague relatively easy to study.

Examination of the *Y. pestis* genome before and after virulence has been induced shows what genes have been turned on. But that information is not enough to show precisely which genes are responsible for various aspects of virulence.

For comparative purposes, a Livermore team led by microbiologist Emilio Garcia collaborated with the Institut Pasteur in France to sequence *Y. pseudotuberculosis*, the parent organism of *Y. pestis*. Although their DNA sequences are about 95 percent identical, *Y. pestis* and *Y. pseudotuberculosis* behave differently. *Y. pseudotuberculosis* lodges in the intestine and causes flulike intestinal distress. *Y. pestis* is also closely related to the mild-mannered *Y. enterocolitica*, an intestinal bug that is itself very much like *Y. pseudotuberculosis*. *Y. enterocolitica* is currently being sequenced by the Sanger Center in Great Britain.

"Bacteria evolve very efficiently and make use of about 80 percent of their DNA," says Fitch. By comparison, humans use only about 30 percent of their DNA. Aiding speedy evolution are the many insertion sequences in a bacterial genome. Insertion sequences are bits of DNA that allow large regions of DNA to replicate themselves and move around the genome, relocating themselves somewhere else. When an insertion sequence lands within a gene, it deactivates that gene. These transfers can also occur across species, and it is not difficult for a bacterium to grab DNA from another bacterium.

Y. pestis evolved from *Y. pseudotuberculosis* within the past 15,000 years, a rapid evolution even for bacteria. "Something happened then to cause *Y. pestis* to learn how to live in a flea," says Garcia.

In addition to their normal chromosomal DNA, bacteria may have smaller circles of DNA known as plasmids. Plasmids replicate separately from chromosomal DNA and often house

From Sequencing to Functional Genomics

DNA decoding, known as sequencing, is the process that determines the precise order of the four nucleic acid bases—adenine (A), thymine (T), guanine (G), and cytosine (C)—that comprise the DNA of all living things. In the case of *Yersinia pestis*, its DNA sequence comprises 4.66 million bases. The Sanger Institute in Great Britain recently published the complete and annotated sequence of the *Y. pestis* genome. For three years before that, a preliminary version of the sequence had been available for use by researchers throughout the world.

As the DNA genome, or parts list, for an organism becomes available—whether it be for plague, mice, or humans—researchers begin to examine the accumulated sequence data very closely. They are trying to identify what makes this particular genome work the way it does—its wiring diagram, so to speak. They search for specific genes. They also study how DNA works with proteins and the environment to create complex, dynamic living systems. Proteins are large molecules composed of amino acids that perform most life functions and make up the majority of cellular structures.

Functional genomics, as this field of research is known, encompasses many topics. Some researchers examine when, where, and under what conditions genes are expressed—that is, turned on. Others study the expression and function of the proteins encoded by certain genes. Still others use x-ray crystallography and other methods to generate three-dimensional structures of proteins, which offer clues to their function. Researchers may inactivate or knock out genes and study the results to learn what specific genes do. Other researchers compare the DNA sequence of several organisms in an effort to identify unique genes and interpret their function. Research is under way in several of these areas as Livermore examines what makes *Y. pestis* so virulent.

genes that encode enzymes critical to the host cell or organism. For example, when a bacterium has become resistant to antibiotic drugs, it is usually because the bacterium has acquired a new plasmid.

One *Y. pestis* plasmid encodes at least two genes that allow *Y. pestis* to survive in fleas. Another plasmid is home to the gene that activates the disease's invasiveness. Researchers have found that *Salmonella* has a similar plasmid, which one bacterium probably obtained from the other.

"The interesting thing is that if you insert the three *pestis* plasmids into *Y. pseudotuberculosis* or *Y. enterocolitica*, you don't get *pestis*," says Garcia. "So something else is going on. Unfortunately, it's never simple."

Once its virulence genes have been turned on, plague infects its host using what is known as Type III secretion, an injection mechanism more colorfully called "*Yersinia's* deadly kiss." *Salmonella typhi*, enteropathogenic *Escherichia coli*, *Chlamydia psittaci*, various species of *Bordetella*, and other pathogenic bacteria appear to share this syringelike injection mechanism. This common trait may indicate another area of transferred genomic material.

Before Livermore's research on plague started, many of the genes critical for virulence had been identified but were poorly understood. The same was true for the underlying mechanisms of virulence. There was also little understanding of the gene and protein interactions that take place between the pathogenic bacteria and its host.

The Pathogen Pathway Project is using functional genomics tools to identify genes important to virulence and understand the pathways of virulence. The team's hypothesized pathway, from DNA to the host organism, is shown in the bottom figure at right.

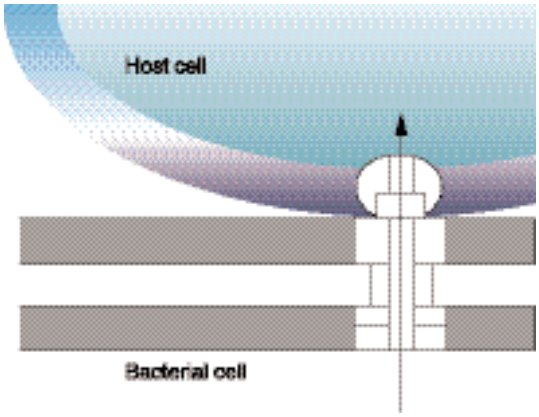
Expression and Function

An early task for Livermore bioscientists and computations experts was to develop a relational database of the DNA sequence of *Y. pestis*. In collaboration with the DOE Genome Consortium at Oak Ridge National Laboratory, these data were used to computationally predict where the 4,500 genes in *Y. pestis* are located and which genes might be associated with virulence.

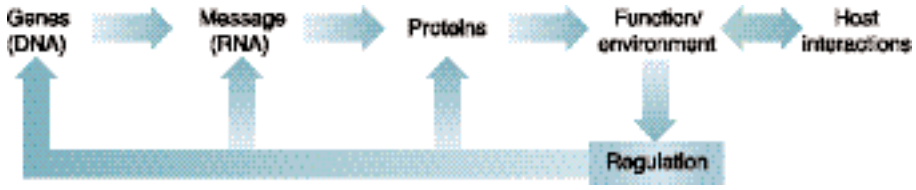
Next, Livermore bioscientist Vladimir Motin and colleagues designed chemical reagents for extracting over 300 genes from *Y. pestis* DNA, including all known virulence-associated genes on the plasmids. In an initial test, they extracted 85 genes associated with virulence and spotted them on a glass microscope slide alongside 11 control spots, making up a 96-spot microarray.

A microarray permits scientists to study the response of thousands of genes or other pieces of DNA quickly and efficiently in a process known as transcript profiling. In the process, each gene receives some kind of stimulus, causing it to turn on and produce messenger RNA (mRNA). In the case of plague, the stimuli are changes in temperature and calcium concentration. The production of mRNA leads, in turn, to the synthesis of unique proteins. The level of mRNA can be measured for each individual gene. The more active or expressed genes there are, the more mRNA will be present.

For the 96-spot microarray, the team developed a protocol to study the response of *Y. pestis* genes under conditions that mimic the infection process: at both flea and human/rodent body temperatures, 26°C and 37°C, and at



The Type III secretion, a syringe-like injection mechanism more colorfully called "*Yersinia's* deadly kiss," which is how plague infects a host once its virulence genes have been turned on.



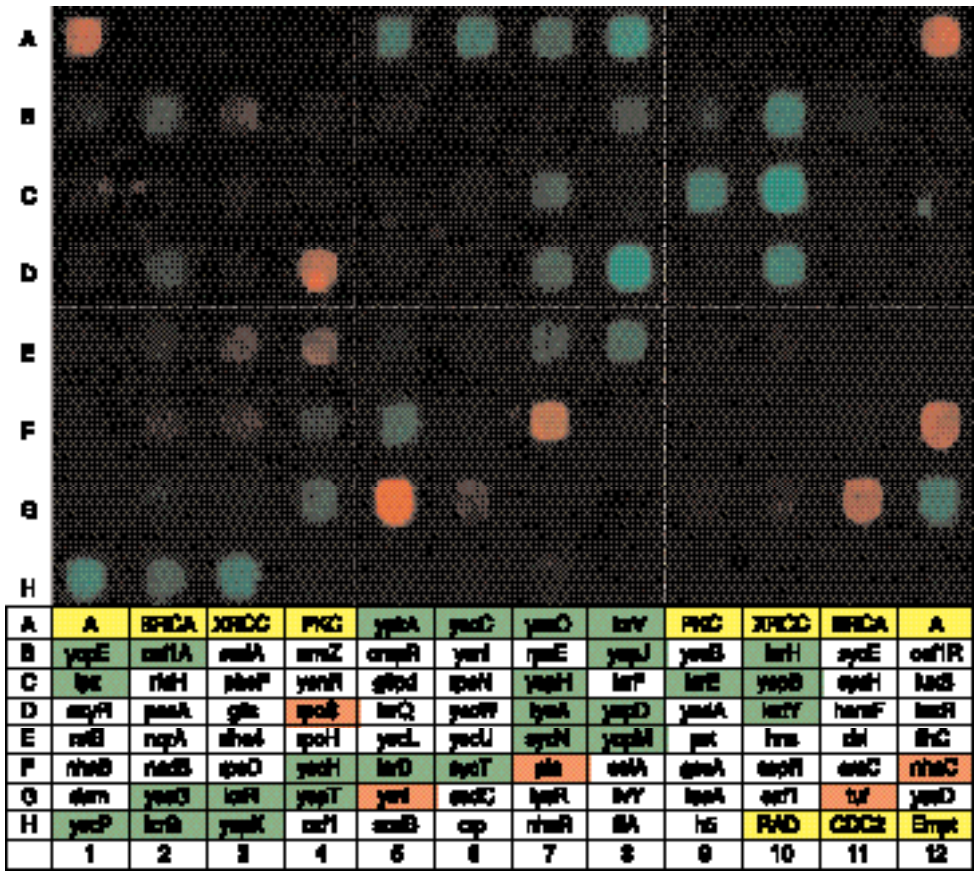
A schematic diagram of information that is hypothesized to describe the pathways of virulence in a pathogen. The regulatory (feedback) loop is often nonlinear, and there can be multiple feedback paths with complex interactions.

calcium levels that correspond to those of blood (higher level) and organs (lower level), the latter location being where more virulence genes are expressed.

More recently, they developed a microarray for all 4,500 *Y. pestis* genes. All of the genes are being mapped at six time intervals as temperature rises and calcium concentration drops. The team is thus beginning to establish a timeline for how and when genes change and are expressed while the plague bacterium is infecting a human host. Some genes are expressed early, while others are late-onset genes. A detailed picture of how the bacterium behaves during the infection process will provide useful information for the development of diagnostic techniques and treatment methods.

Garcia and other researchers also completed a detailed analysis of three *Y. pestis* plasmids, which allowed them to confirm the location of several known virulence genes and to uncover four novel ones believed to contribute to virulence. Computerized comparisons with other genomic databases indicated the presence of a large number of virulence-related genes that are similar in both closely related bacteria such as *Y. pseudotuberculosis* and distantly related bacteria such as *E. coli*. The team also found numerous gene coding regions whose function they could not determine.

Using a proteomic approach of protein separation techniques and mass spectrometry (MS), Livermore researchers led by Sandra McCutchen-Maloney are analyzing complex mixtures of proteins isolated from *Y. pestis*. By comparing samples grown at the two physiological conditions mimicking the flea and the human (at 26°C and 37°C, respectively) and at low calcium concentration to induce virulence, the team is detecting differential protein expression to identify candidate proteins important for *Y. pestis* pathogenicity. Comparisons are also being made between human cells that have and have not been exposed to *Y. pestis* in order to understand the host immune response. Because it is the proteins that are actually responsible for virulence effects, the group is also working to correlate their proteomic data with genomic data obtained from microarray experiments. To learn more about the individual proteins responsible for virulence, the team is using various biochemical assays to test functional models of the candidate virulence factors. In addition,



A 96-spot microarray for transcript profiling of 85 genes of *Yersinia pestis*; 11 of the spots are controls. Genes tagged with fluorescent dyes change color in response to various stimuli.

McCutchen-Maloney's group is looking at host–pathogen interactions by using surface-enhanced laser desorption ionization (SELDI) MS to study various protein–DNA and protein–protein interactions within *Y. pestis* and between *Y. pestis* and the human host. For example, regulatory proteins that bind to genes and control differential expression are under investigation, as are the specific protein–protein interactions of suspected virulence factors. These molecular interactions are key to the genetic feedback that occurs as a pathogen infects its host, as shown in the bottom figure on p. 7.

Differences Are Key

Before Garcia's team completed its comparative sequencing of *Y. pseudotuberculosis*, microbiologists Gary Andersen, Lyndsay Radnedge, and others examined the differences between *Y. pseudotuberculosis* and *Y. pestis* using a different technique. This process, developed in Russia, is known as suppression subtractive hybridization (SSH). SSH identifies regions of DNA that are present in one species but absent in another.

SSH has the advantage of requiring only small amounts of genomic DNA. It can be used with any genome, even one that has not yet been characterized. It is especially useful for identifying the large genomic differences typically found between bacterial genomes. For example, SSH identified the genetic material that causes Kaposi's sarcoma, a skin lesion associated with HIV and AIDS. At Livermore, SSH has been useful for finding differences among anthrax strains and other potential agents of bioterrorism.

Comparison of *Y. pestis* and *Y. pseudotuberculosis* revealed seven DNA regions in *Y. pestis* that do not occur in *Y. pseudotuberculosis*. Four of them occur very closely to one another on the *Y. pestis* genome. "It is fair to assume that *pestis* acquired this region during its evolution from *Y. pseudotuberculosis,*" says Radnedge.

To learn more about the function of genes in these areas, Garcia and others are beginning "knock-out" studies. They will inactivate, or knock out, one gene at a time and test the resulting bacterium on an animal to see how the host and its genes respond. This is slow, laborious work, but it will help to determine what the function of each *Y. pestis* gene is, if any, and what gene or genes in the host are expressed as a response. This detailed examination of pathogen–host interaction for plague will be the first of its kind.

Being Prepared

Research to date on plague lays the groundwork for additional work planned at Livermore in the areas of microbiology, proteomics (the global study of proteins), bioinformatics (the integration and analysis of biological data), and biological modeling for the NNSA's Chemical and Biological National Security Program. Some of the research will elaborate on plague, some will examine a broader spectrum of human pathogens, and some will further the development and use of biodetectors, mass spectrometry, and other technologies.

In the U.S. today, plague pops out of the rodent population and into the human populace occasionally in the desert Southwest. It is a larger problem in a few other countries. But the real fear is that plague could be used as an agent of mass destruction. At least in industrialized countries, it is unlikely that plague would cause the huge number of deaths that occurred during earlier epidemics. Better sanitation, a more educated populace, and a far superior medical system would likely prevent that. But the world needs to be prepared.

—Katie Walter

Key Words: bioterrorism agents, Chemical and Biological National Security Program, plague, surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS), suppression subtractive hybridization (SSH), virulence, *Yersinia pestis*, *Yersinia pseudotuberculosis*.

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L-Gel Decontaminates Better Than Bleach

Scientists have developed a material that is safe for people and the environment but deadly to the agents of biological and chemical warfare.

THE recent cases of anthrax spores deliberately spread through the mail reminded all Americans, and especially managers of federal and state agencies responsible for public health and safety, about potential terrorism with chemical and biological weapons. The anthrax cases have also underscored the need for safer and more efficient methods to decontaminate offices and homes of deadly biological agents.

During the late 1990s, scientists at the Department of Energy national laboratories foresaw the need for a safe, reliable, and easily deployable decontaminating agent that could be used for civilian defense against biological and chemical terrorism. DOE managers agreed with the scientists and asked them to use their expertise in chemistry, biology, and environmental protection to develop new decontamination products and procedures.

Lawrence Livermore responded to this request with a team formed from the Environmental Protection Department and three directorates—Chemistry and Materials Science; Nonproliferation, Arms Control, and International Security; and Biology and Biotechnology Research. The team of diverse experts developed a compound called L-Gel (the L is for Livermore), which combines a mild, commercially available oxidizer with a silica gelling agent to create a substance that coats walls, ceilings, and other materials like a paint, effectively decontaminating the coated surface.

The material is nontoxic, noncorrosive, easy to manufacture, easily deployable, and relatively inexpensive (about $1 to cover a square meter). Tests at Livermore's laboratories and field trials at both federal and foreign facilities have shown that L-Gel has been extremely effective at decontaminating all classes of chemical warfare agents as well as surrogates for biological warfare agents.

Livermore technology transfer specialists are currently engaged in negotiations with several companies to license the manufacturing and marketing of L-Gel. If negotiations proceed apace, government agencies could have the material by the end of the fiscal year (September 30) to respond to any terrorist incident involving chemical or biological agents.

Different Needs for Civilians

According to L-Gel development leader Ellen Raber, a geochemist and head of Livermore's Environmental Protection Department, several decontaminating agents are effective against either chemical or biological warfare agents. However, these materials, which are mainly strong chemicals, were developed by the military for battlefield use, and they pose environmental and health risks when used in civilian settings. At the minimum, they can damage everyday materials such as furniture and office equipment.

Other methods that have been used in civilian settings have serious drawbacks. For example, solutions of laundry bleach work well as decontaminants but are very corrosive. Incineration and irradiation have obvious practical limitations in office settings or face public resistance. Chlorine dioxide gas, used late last

year to decontaminate the Hart Office Building that houses members of the U.S. Senate, is a laborious process and poses a safety risk to workers. It also requires the gassed building to be neutralized before people can reenter.

The Livermore team focused on finding an effective decontaminating agent and application system that is safe to use, does not damage commonly used materials and surfaces, is friendly to the environment, and is effective against both chemical and biological warfare agents. "We wanted something that was less corrosive than bleach, that is easy to apply, and that does not leave workers with a huge cleanup job," Raber says.

Raber points out that speed of decontamination, which is all-important in military applications, is less important in civilian applications, where decontamination times of one to several hours may be adequate. More important in a civilian scenario are ease of application, minimal training required for use, moderate expense, and environmentally acceptable byproducts.

The team also recognizes that the new product needs to be effective in three potential settings of a terrorist incident against civilians: an outdoor location such as a stadium, a semienclosed place such as a subway station, and an enclosed space such as an office building. Using the decontaminating material on interior surfaces can have quite different requirements from those appropriate for outdoor use, where natural attenuation from environmental conditions (for example, ultraviolet radiation from sunlight) might well be adequate for effective decontamination.

Start with the Oxidizer

The development effort began with Livermore scientists Ray McGuire and Don Shepley evaluating several acidic oxidizer solutions that could degrade chemicals into nontoxic, environmentally acceptable components. (Oxidizing solutions do not completely destroy chemical agents but rather break key chemical bonds to render the toxic compound inactive.) The oxidizers considered could be deployed in liquid spray systems or incorporated into compatible gels for clinging to surfaces such as ceilings and walls.

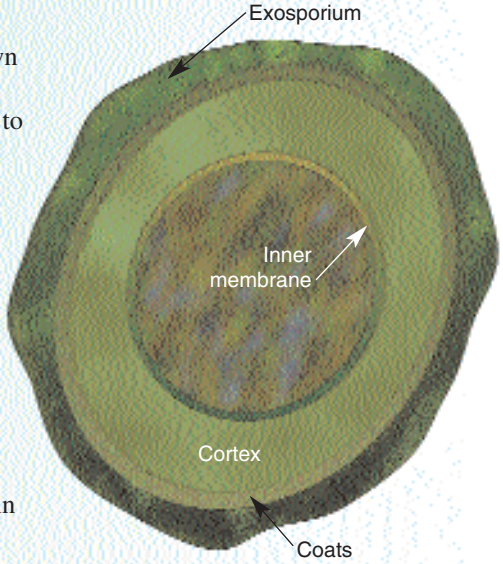
McGuire chose an acidic rather than a basic oxidizer solution, primarily to aid the decontamination of VX, a potent nerve agent. Acidic oxidizer solutions are also known to be effective at decontaminating certain biological warfare agents, including bacterial spores, which are extremely difficult to kill because of their hard, multilayered coats. The coat allows a spore to remain in a dormant state for many years until, under the right environmental conditions, it transforms into a live organism.

"Anthrax is the most difficult biological agent to kill because of its resistant outer coat," says Raber. An oxidizer in acidic solution breaks down the proteins that are found in anthrax coats. Once the oxidizer gets through to the nucleus, its molecules destroy strands of the anthrax DNA or RNA.

The goal was to find the most effective oxidizer at the lowest effective concentration. The oxidizers that were evaluated included potassium permanganate, peroxydisulfate, peroxymonosulfate, hydrogen peroxide, and sodium hypochlorite. The oxidants were evaluated in laboratory tests on chemical warfare surrogates for such agents as VX, sarin (used in the Tokyo subway terrorist incident), and sulfur mustard (used during World War I).

Livermore bioscientist Paula Krauter evaluated the same group of oxidizers on surrogate biological agents and toxins that would likely be used in terrorist attacks. *Bacillus subtilis* was used for spore-forming agents such as anthrax, *Pantoea hericola* was the surrogate for plague, and ovalbumin was the surrogate protein for botulinum toxin.

The initial laboratory tests showed that potassium peroxymonosulfate was more than 99 percent effective at oxidizing both chemical and biological warfare surrogates that were placed on common materials such as carpet, wood, and stainless steel. The results led to the selection of Oxone, a commercial product manufactured by DuPont, which contains potassium peroxymonosulfate—its active ingredient—in a water solution. Previous research at U.S. military laboratories had demonstrated the effectiveness of Oxone in decomposing both VX and mustard-type agents, but the compound had not been previously tested on biological agents.



The cross section of a bacterial spore, such as anthrax, shows its hard, multilayered coats, which both make the spore difficult to kill and allow it to remain dormant for many years.

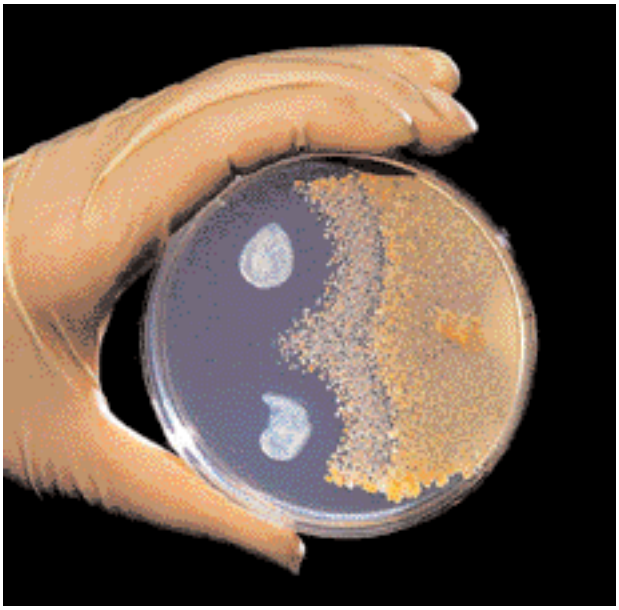
Gel Adds Staying Power

The team recognized that spraying water-based solutions of Oxone would not be effective in all cases. Consequently, McGuire and Mark Hoffman investigated carrier materials that would thicken the oxidizer so it would better cling to walls, ceilings, and other surfaces to increase contact time with the biological or chemical agent.

Hoffman chose colloidal amorphous silica as the carrier material for several reasons. First, unlike crystalline silica, which is toxic, colloidal amorphous silica is safe to use and is found in many household paint formulations. Also, silicon dioxide colloidal particles are commercially available, don't require manufacturing in a special facility, and, because they are chemically inert, are compatible with oxidant solutions. When mixed with the oxidizer, the gel can be applied with simple delivery systems, such as paint sprayers. After application, it thickens and tends not to sag or flow down walls or drip from ceilings. Finally, silica gel materials can be easily vacuumed up after they have dried.

Livermore chemists have extensive experience with colloidal silica gel. From the late 1960s to the late 1980s, the chemists developed a series of extrudable high explosives based on the gelling of energetic liquids. Although this research did not advance to the explosives production stage, the development effort provided useful experience for working with silica-gel materials. It was a logical step to adapt this work to the gelling of aqueous oxidizers for candidate decontaminants, says Hoffman. "Our research with high explosives gave us a good feel for working with silica gels."

Hoffman selected Cab-O-Sil EH-5 fumed silica as the gelling agent. The final formulation was named L-Gel 115, which is a formulation of aqueous Oxone solution gelled with 15 percent EH-5 silica gel. The viscosity can be varied, depending on the application. Under development is a second formulation, called L-Gel 200, which contains 10 percent t-butanol cosolvent to promote penetration on surfaces with heavily coated paint or varnish.



The biocidal effect of peroxymonosulfate, the oxidizer in L-Gel, is seen on this nutrient agar plate of *Bacillus subtilis* spores (surrogates for anthrax). Three spots of silica gel were added to the plate. Two of the spots contained peroxymonosulfate and one (at right) did not. The peroxymonosulfate-containing gel inhibited spore germination in the zone surrounding the gel, even leaching into the agar.

Field Tests Prove Effectiveness

The final L-Gel 115 formulation was subjected to a series of tests at Livermore facilities using surrogates of potential terrorist chemical and biological agents. The tests involved placing surrogate chemical and biological agents on various common materials—varnished wood, painted steel, glass, fiberglass, and carpet—adding L-Gel to the surface, allowing the gel to dry for 30 minutes to several hours, and then determining the percentage of surrogate that had been decontaminated. L-Gel proved greater than 99 percent effective on all surfaces and for all agents.

The Livermore biological researchers also tested L-Gel on safe strains of the deadly biological agents *Bacillus anthracis* (anthrax) and *Yersinia pestis* (plague). These strains—Sterne and Strain D27, respectively—could be safely used in experiments because they are nonvirulent, that is, they do not contain the genes that create the lethal toxins present in the real organisms. (See the article beginning on p. 4 about research on sources and pathways of virulence in organisms.) The researchers used the agar plate resistance test, a standard technique to measure the efficacy of antibiotics. In this test, about one million cells (or spores, in the case of *B. anthracis*) were combined with liquid agar, then poured onto a petri dish containing nutrients for cell growth. The strains were also tested against dilutions of L-Gel, which proved more than 99.9 percent effective in killing the cells and spores.

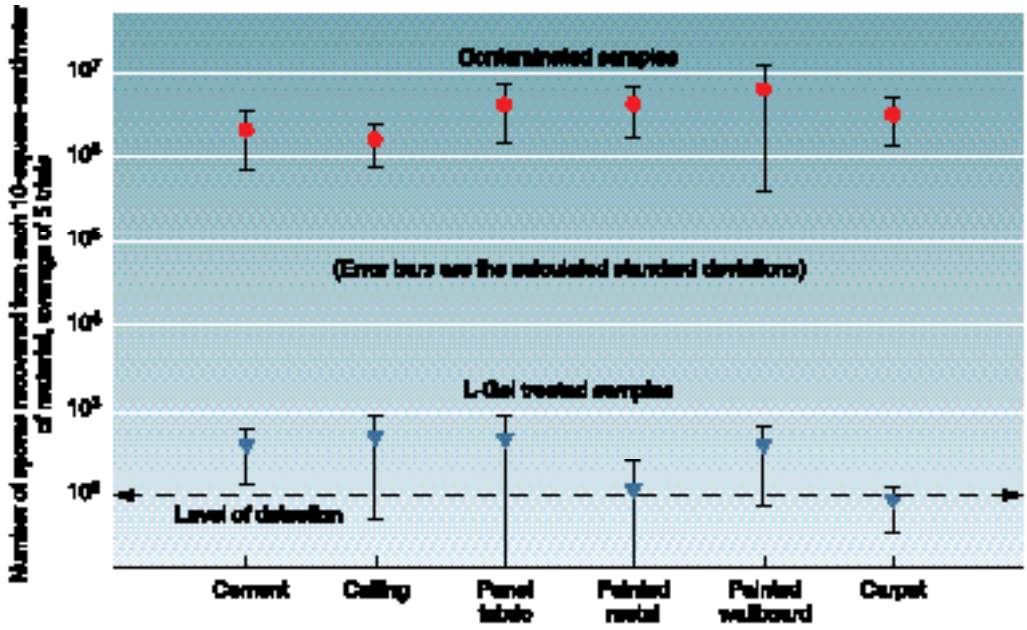
L-Gel also was tested against surrogate spore-forming bacteria in two field exercises. In December 1999,

researchers Krauter and Tina Carlsen participated in biological warfare field tests that were conducted by the Soldier Biological and Chemical Command at the U.S. Army Dugway Proving Ground, Utah. The tests compared the ability of several decontamination materials to inactivate surrogate organisms placed on six 40-square-centimeter panels of acoustic ceiling tile, tightly woven carpet, fabric-covered office partition, painted wallboard, concrete slab, and painted metal. Each panel was contaminated with about 10 billion spores per square meter.

After L-Gel was applied, the panels were swabbed about 24 hours later. The number of live spores on most test panels was reduced by an average of 99.988 percent.

In October 2000, Krauter and Hoffman participated in a biological warfare agent room-decontamination exercise that was conducted again at the Dugway Proving Ground. The tests used full-scale, mock offices constructed in an abandoned building. Flooring was divided into quarters consisting of carpet, vinyl tile, varnished oak, and painted concrete. Walls consisted of stucco, wood paneling, plasterboard, and carpet, and the ceiling was constructed of suspended ceiling tile. The room was contaminated with 4 grams of spores. After application of L-Gel, about 400 samples were collected from multiple locations in the room. L-Gel reduced the number of spores by about five orders of magnitude and, in these experiments, did not damage

Researcher Paula Krauter applies L-Gel to "contaminated" panels of different materials to test the gel's effectiveness.



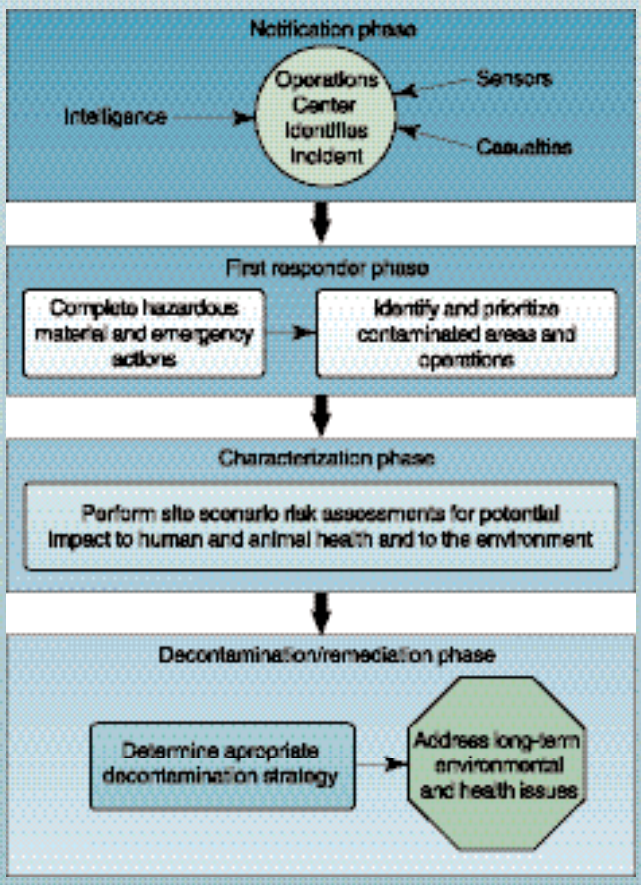
L-Gel was tested against surrogate spore-forming bacteria at the Soldier Biological and Chemical Command at the U.S. Army Dugway Proving Ground, Utah. In one test, surrogate organisms were placed on six 40-square-centimeter panels of acoustic ceiling tile, tightly woven carpet, fabric-covered office partition, painted wallboard, concrete slab, and painted metal. L-Gel reduced the number of live spores on most test panels by an average of 99.988 percent.

How Clean Is Clean Enough?

When a terrorist attack on civilians potentially involves biological or chemical warfare agents, decision makers will need to make fast and informed choices about how to respond. A team of Livermore researchers from the Safety, Security, and Environmental Protection Directorate has developed a process that guides users to make the best emergency response decisions involving notification, identification, characterization, decontamination, and cleanup.

In 1998, at the request of DOE's Office of Nonproliferation and National Security, the team developed a biological agent decontamination plan in the form of a flowchart. It was then used in a recommendation from the Environmental Protection Agency to the National Security Council, the President's principal forum for considering national security and foreign policy issues. In 2001, the Livermore team added chemical warfare agents to the plan, now termed the Chemical and Biological Agent Decision Process.

The process helps users to determine what actions need to be taken at the outset; if an actual or potential impact to health, property, or the environment exists; whether or not decontamination is needed; what steps should be taken and when; and how to verify that cleanup and remediation are complete so that the area can be designated as safe to reenter or reuse.



Under the process, each of four phases (notification, first responder, characterization, and decontamination/remediation) progresses to the next phase as soon as all its issues have been addressed. The format includes numerous yes/no decision points and links to more detailed information on specific topics. The decision process takes into account different environments, such as an outdoor site, and considers individuals in the general population who may be at higher risk for illness and injury.

According Ellen Raber, head of Livermore's Environmental Protection Department, the biological agent decontamination plan addresses a need by several federal agencies for an up-to-date summary of information necessary to evaluate acceptable decontamination levels and procedures. The goal of the plan is to help minimize the number of deaths and illnesses, damage to the natural and built environment, and the extent of economic damage (for example, crop or livestock damage) resulting from a biological terrorism incident.

In developing the plan, Livermore experts did a thorough literature search and consulted with colleagues at the U.S. Army, the U.S. Environmental Protection Agency, and federal health agencies, including the U.S. Public Health Service. The team noted that responding to a civilian event involves different priorities than those for a military setting. For example, during a battle, quick decontamination is critical so soldiers can continue their mission. In a domestic urban scenario, however, considerations of public health and environmental issues are usually more important than immediate decontamination. Also, the decontamination process may need to be staged, with cleanup of gross contamination—for example, of puddles of toxic materials—followed by more localized decontamination, such as cleaning up materials in cracks.

The flowchart is structured so that cleanup criteria are dependent upon the decontamination site. Much stricter criteria are necessary for indoor settings such as offices or homes than for outdoor scenarios where wind, sunlight, temperature, and rain may effectively decontaminate biological agents, toxins, and chemical warfare agents.

Raber says the decision process must include answering the question, "How clean is clean enough?" In this respect, it is more difficult to establish target cleanup levels for biological agents than for chemical agents, in large part because of public acceptance and perception issues. She notes that the public may demand zero living organisms after decontamination, but achieving such a level may not be practical or necessary. In the case of anthrax, for instance, it takes about 6,000 inhaled anthrax spores to cause respiratory anthrax. Furthermore, some biological agents such as anthrax are already indigenous to many farming communities and exist without incident. "Zero concentration of a biological agent and zero risk, in many cases, are clearly not a necessity," she says.

Raber also points out that it is possible to do a poor job of decontamination and to make it look good by doing a poor job of sampling and analysis. "In the end, decontamination must be defensible to regulatory agencies and to the public."

office surfaces, with the exception of bleaching some rust on ceiling supports.

L-Gel was also independently tested on real chemical warfare agents at four locations from October 1998 to October 2000. The tests were conducted at the Military Institute of Protection, Brno, Czech Republic; Edgewood Chemical and Biological Forensic Analytical Center, Maryland; the Defense Evaluation and Research Agency, United Kingdom; and the Soldier Biological and Chemical Command at Dugway. Field tests showed that L-Gel was a more effective decontaminant of real VX, GD (nerve agent), and sulfur mustard than the current military standard, calcium hypochlorite, on such materials as acrylic-painted metal, polyurethane-coated oak flooring, and indoor–outdoor carpet.

Two of the field trials also demonstrated that the L-Gel 200 formulation has improved penetration and thus promotes solution and oxidation in thickened chemical agents. L-Gel 200 was tested on real chemical warfare agents such as thickened distilled mustard and thickened soman (persistent nerve agent) as part of the Restoration of Operations series of experiments at Dugway Proving Ground. The agents were applied on steel test panels, Air Force air–ground equipment paint, and Navy shipboard coating.

Meets Safety Standards

With L-Gel's excellent performance demonstrated in both laboratory and field trials, it was time to partner with one or more commercial firms that could manufacture the material quickly and efficiently. Fortunately, says Raber, "L-Gel is simple to manufacture. It's comparable to mixing paint." L-Gel is relatively noncorrosive (its pH is about 4, similar to that of vinegar or lemon juice), and Environmental Protection Agency testing shows its residual materials to be nonhazardous. It also meets the Department of Transportation's nonhazardous and noncorrosive requirements and is stable during shipping.

L-Gel is premixed and then shipped and stored as a semisolid resembling Jello at room temperature. If unopened, its shelf life is expected to exceed a year. It is reliquefied to the consistency of house paint by vigorous shaking by hand or a power stirrer. It can be applied with any type of commercially available spray device, whether airless or compressed-air units, with any stainless-steel atomizing nozzle.

Although L-Gel clings to walls and ceilings, it does not harm most painted surfaces or carpets. Decontamination takes about 30 minutes. When dry (in about 1 to 6 hours), the gel residue, unreacted oxidizer, and decontaminated chemical or biological agents can simply be vacuumed up and discarded as nonhazardous waste. For outdoor use, no cleanup is required.

Raber says L-Gel compares favorably to other decontamination methods that have been used recently to kill anthrax spores. The tried-and-true method is a bleach solution. However, bleach is extremely corrosive to metal surfaces and must be used with care by cleaning crews.

A foam developed at Sandia National Laboratories in New Mexico has also been effective for decontaminating chemical and biological agents. This material is sprayed on surfaces like a firefighting foam. Most of the foam dissipates, and the residual material is then washed off. It has been used to clean offices of Congress and at ABC News. Raber suggests that L-Gel and the Sandia foam could work in tandem, with L-Gel sprayed on walls and ceilings and the Sandia foam applied to large pieces of equipment and floors.

Chlorine dioxide, used to decontaminate U.S. Senate offices, is a gas that kills bacteria but also is hazardous to human health and thus must be applied by trained personnel. Afterward, its vapors must be sucked out of rooms and then filtered through an ascorbic acid bath to decompose it. Raber notes

A second test at the Dugway Proving Ground in Utah tested L-Gel in a mock office setting.

L-Gel is shipped premixed as a semisolid. It is reliquefied to a house-paint consistency by vigorously shaking it by hand or using a power stirrer.

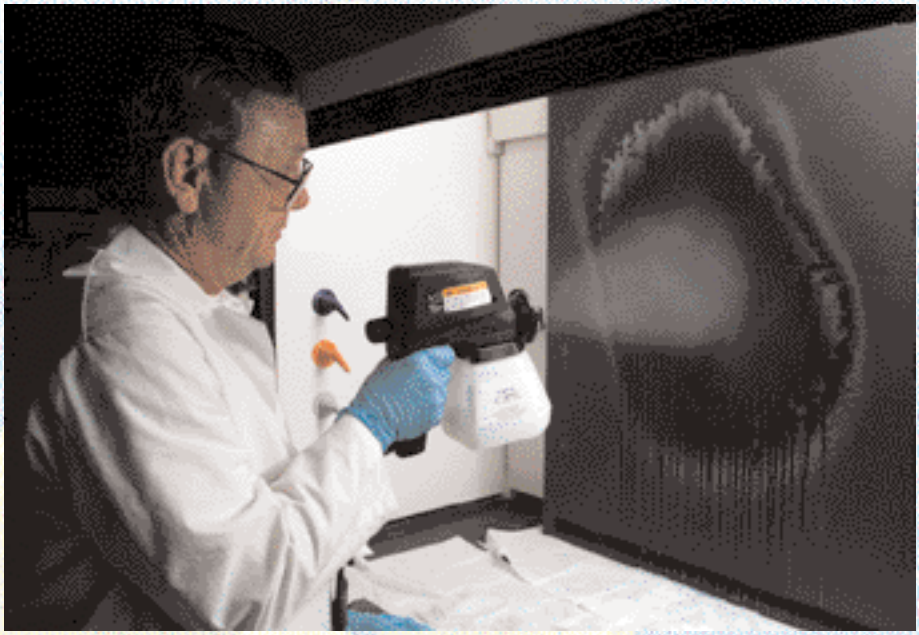
that gases and aerosols have clear advantages for decontaminating ventilation systems and hidden spores, and research needs to continue to find an environmentally safe gas or aerosol that is effective for these applications.

Irradiation, popular in Europe, kills bacteria and spores and is effective in decontaminating mail, food, and other objects. However, the method requires large machines, which are essentially small accelerators, and is not currently viable for large-scale room decontamination.

In the News

News about L-Gel has spread rapidly, and Raber has been interviewed by several newspapers, television stations, and National Public Radio. She has also received a large number of inquiries from emergency response groups across the country interested in additional information and samples.

The developmental work for L-Gel 115 is complete, and Raber's team has begun to develop a new formulation to decontaminate ventilation systems. "Right now, we don't have an easy way to decontaminate air ducts," she says. The team is working on an encapsulation method to aerosolize L-Gel (make it into tiny droplets) so that it could be blown into ventilation systems.



Livermore chemist Mark Hoffman uses a household paint sprayer to apply L-Gel to a test panel.

In the meantime, licensing of L-Gel manufacture is well under way, and Raber is hopeful that major organizations will soon have an important yet nontoxic new weapon to counter any biological or chemical attack.

—Arnie Heller

Key Words: anthrax, biological warfare, Chemical and Biological Agent Decision Process, chemical warfare, decontamination, L-Gel, peroxymonosulfate.

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Rapid Field Detection of Biological Agents

FOR years, experts in terrorism have been warning that a terrorist attack with biological agents is not a question of "if" but "when." As recent events have proved, when is now.

For almost a decade, researchers at Lawrence Livermore, working on the when-is-now premise, have been developing systems that can rapidly detect and identify biological agents, including pathogens such as anthrax and plague. (For more background on Livermore's research against bioterrorism, see *S&TR*, June 1998, pp. 4–9, and May 2000, pp. 4–12.) Among such systems are the Handheld Advanced Nucleic Acid Analyzer (HANAA) and the Autonomous Pathogen Detection System (APDS).

The Handheld Advanced Nucleic Acid Analyzer can detect biological pathogens in the field.

Lawrence Livermore National Laboratory

Although HANAA and APDS are of different sizes and made for different situations, they have a common purpose: to get results, fast. Lawrence Livermore biological scientist Richard Langlois explains, "There are any number of laboratory tests available right now to analyze pathogens. They all require getting a sample and then transporting it to a laboratory for processing. Our systems use new instrumentation and methods that provide faster and more timely results, on the spot. Faster results mean the responders can act quickly and begin treatment earlier."

HANAA in Hand

About the size of a brick, the HANAA biodetection system can be held in one hand and weighs less than a kilogram. The system was designed for emergency response groups, such as firefighters and police, who are often first on the scene at sites where bioterrorism may have occurred. Each handheld system can test four samples at once—either the same test on four different samples or four different tests on the same sample. HANAA can provide results in less than 30 minutes, compared with the hours to days that regular laboratory tests typically take.

The process of detecting and identifying what's in a sample works like this. The operator prepares the samples by putting them in a liquid buffer and adding chemicals. A tiny disposable plastic tube holding about 0.02 milliliter of the prepared liquid is then inserted into the system. Many copies of a sample's DNA are needed to analyze it and identify its makeup. HANAA uses a technique called the polymerase chain reaction (PCR), which amplifies agent-specific DNA fragments to a detectable level. In PCR, an aqueous sample is heated close to the boiling point and then cooled many times (40 times in HANAA). Every time the DNA is heated, the two intertwined strands of DNA unwind and come apart. As the sample cools down, the DNA makes a copy of itself. Thus, at the end of each cycle, the amount of DNA is doubled.

To detect the DNA in a sample, a synthesized DNA probe tagged with a fluorescent dye is introduced into the sample before it is inserted into the heater chamber. Each probe is designed to attach to a specific organism, such as anthrax or plague. Thus, the operator must have an idea of what substances might be involved. "The system doesn't test for all unknowns," says Langlois. "A responder has to decide what kinds of pathogens to test for ahead of time and set up the system accordingly." If that organism is present in the sample, the probe attaches to its DNA, which is then amplified during the PCR process, releasing the fluorescent tag. HANAA measures

the sample's fluorescence and the presence (or absence) of the targeted organism.

One of the big breakthroughs for the handheld system involved the design of a small silicon heater chamber for the heating and cooling cycle, a concept developed at Livermore by Allen Northrup, a former Laboratory scientist. "The commercial thermocyclers used for standard laboratory tests are pretty big, ranging from the size of a microwave oven to a large desk," notes Langlois. "A typical large thermocycler takes about 3 minutes to cycle through one heating and cooling cycle, so a complete analysis requires 2 to 3 hours." In the HANAA system, the thermal cycling process occurs in tiny silicon heater chambers, micromachined by Livermore's Center for Microtechnology. Each chamber has integrated heaters, cooling surfaces, and windows through which detection takes place. Because of the low thermal mass and integrated nature of the chambers, they require little power and can be heated and cooled more quickly than conventional units. The mini-chambers typically cycle from about 55°C to 95°C and back to 55°C in about 30 seconds.

Using this technique, the HANAA system could, in principle, detect as few as 10 individual bacteria in one-hundredth of a milliliter in less than 30 minutes. The system has the potential of saving many lives by saving time—anthrax, for example, is highly treatable if detected early.

The Laboratory has a cooperative research and development agreement for HANAA with Environmental Technologies Group (ETG), a chemical and biological detector company and subsidiary of Smith's Industries, based in Baltimore, Maryland. ETG expects to have a commercial version of HANAA available early this year. Ron Koopman, special projects manager for the Chemical and Biological National Security Program at Livermore, notes that HANAA is essentially ready to go at this critical juncture because of the forward-thinking efforts begun in the previous decade. "A number of people recognized the vulnerability of the country to bioterrorism a long time ago," he says. "In 1996, although bioterrorism seemed far away and was something we hoped would never happen, the Laboratory and members of the defense community decided to invest in the research, just in case. Thanks to that investment, we now have something to put in the hands of people to protect us all, something that can help during the current crisis and in the long run."

A Bio "Smoke Detector"

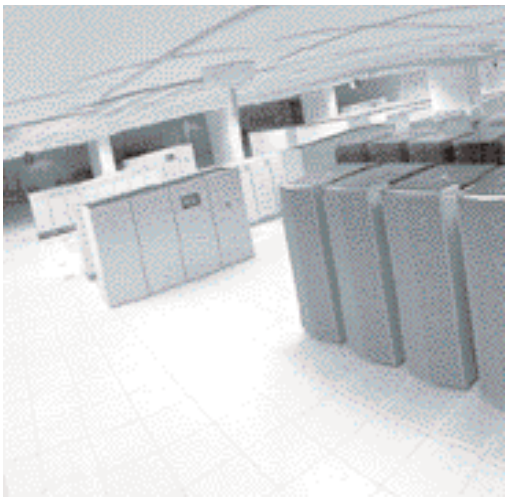
Whereas HANAA can be hand-carried to sites at which an attack is suspected to have happened, the APDS is stationed in one place for continuous monitoring and is designed to work much like a smoke detector, but for pathogens. When fully developed, the APDS could be placed in a large area such as an airport, a stadium, or a conference hall. The system will sample the air around the clock and sound an alarm if pathogens are detected.

"The important point here is that the system would be fully automated," stresses Langlois. "The system will collect and prepare the samples, do the analysis, and interpret the results, all without human assistance."

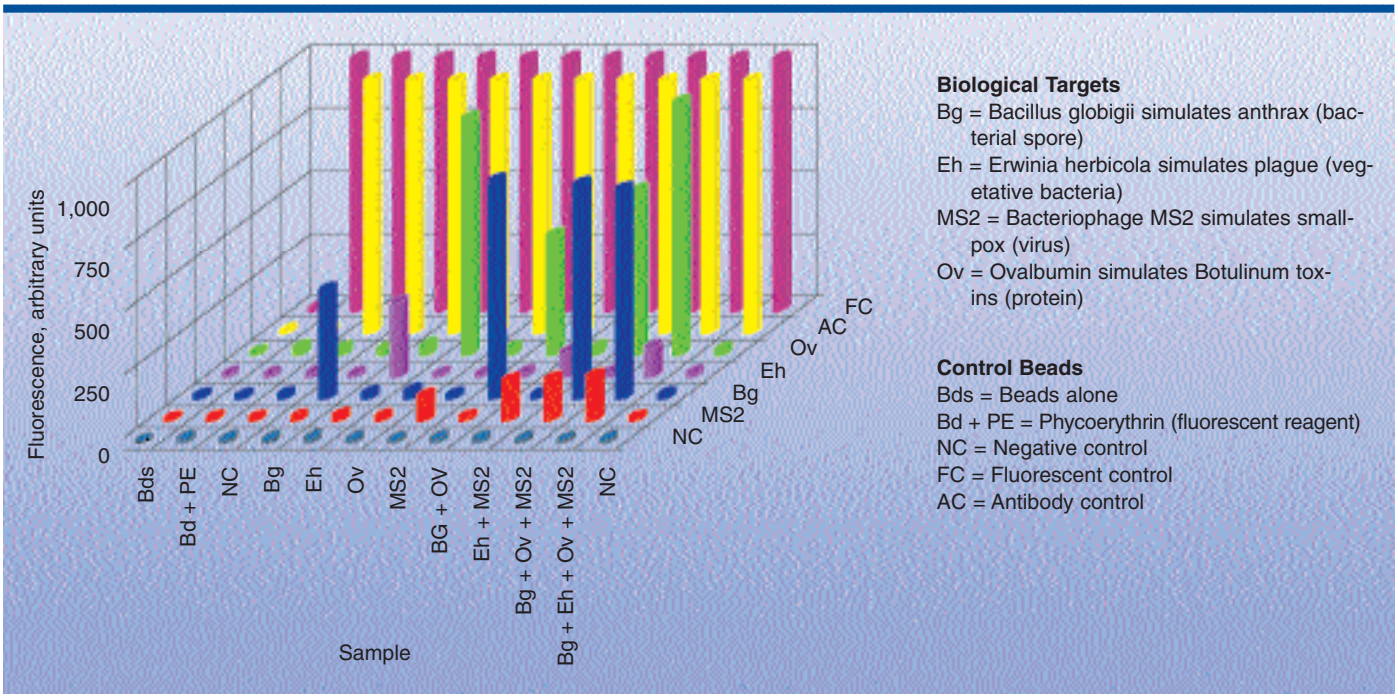
Livermore is testing the second APDS prototype, which is about the size and shape of a lectern or mailbox. The APDS-II consists of an aerosol collector, a sample preparation subsystem, and two subsystems for detecting and analyzing the samples: one based on PCR and the other based on flow cytometry, which uses antibodies to identify pathogens. "The final system will double-test each sample to decrease the likelihood of false positives and increase the reliability of identification," explains Langlois.

The aerosol collector, which was designed by Vern Bergman and Don Masquelier at Livermore, gathers an air sample every 30 minutes—the length of time it takes to complete a sample analysis. A built-in fan pulls in the air, which passes through a glass tube containing water. The water traps any particles in the air, and the resulting fluid is pumped to the next stage for sample preparation and testing.

The flow-through PCR subsystem for the APDS includes a Livermore-designed thermocycler—much like the thermocycler in HANAA—along with a sequential injection analysis system. This analysis system performs all the necessary PCR sample preparation functions, such as mixing the sample with PCR reagents, delivers the resulting liquid sample to the thermocycler, and decontaminates the thermocycler chamber and fluid delivery tubes to prepare for the next run.



The Autonomous Pathogen Detection System is capable of continuous, automated, 24-hour monitoring for pathogens, with results reported every 30 minutes.



Results from the flow-cytometry subsystem of the Autonomous Pathogen Detection System (APDS) using seven color-coded "capture" beads coated with antibodies specific to the target pathogens. The pathogens attach to their respective antibodies and then to more antibodies added to the sample mixture that are labeled with fluorescent dyes. When the beads pass one by one through the flow cytometer's laser beam, any bead with labeled antibodies will fluoresce, and the APDS identifies the pathogens present, depending on the color of the capture bead.

For the flow-cytometry subsystem, small "capture" beads that are 5 micrometers in diameter are coated with antibodies specific to the target pathogens. The beads are color coded according to which antibodies they hold. Once the pathogens attach to their respective antibodies, more antibodies—those labeled with a fluorescent dye—are added to the mix. A labeled antibody will stick to its respective pathogen, creating a sort of bead sandwich–antibody, pathogen, and labeled antibody. The beads flow one by one through a flow cytometer, which illuminates each bead in turn with a laser beam. Any bead with labeled antibodies will fluoresce. The system can then identify which agents are present, depending on the color of the capture bead. "Right now, we use seven bead types to detect four agents simultaneously with controls," says Langlois. The next step is to increase the number of detectable pathogens to 20 or 30. Ultimately, the researchers expect to be able to test for a hundred pathogens simultaneously in a single assay.

Langlois and the APDS team hope that, within the next year or two, the system will be ready to put in place wherever needed. Ultimately, notes Langlois, numerous detector systems could be linked together in a network connected to an emergency response center to protect a complex of buildings or a city.

The Faster the Better

From handheld, immediate testing to autonomous and continuous testing, HANAA and APDS are two of many systems Livermore is developing to help the nation fight bioterrorism. With HANAA, emergency responders can get answers on the scene in less than half an hour. With APDS, no human direction will be necessary, and the system will perform on its own, completely self-contained, monitoring 24 hours a day, 7 days a week. "What ties these approaches together is the ability to analyze a sample quickly—within 30 minutes or less—and do it on site," concludes Langlois. "Getting the answer quickly is important. In the case of a biological attack, the sooner we know what bioagent we're dealing with, the sooner treatment can start for those affected. Systems such as these have the potential for saving many lives."

—Ann Parker

Key Words: anthrax, Autonomous Pathogen Detection System (APDS), biodetectors, biological warfare agents, bioterrorism, DNA analysis, flow cytometry, Handheld Advanced Nucleic Acid Analyzer (HANAA), pathogens, polymerase chain reaction (PCR).

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When Lethal Agents Rain from the Sky

CONSIDER this: a ballistic missile carrying a chemical or biological agent is traveling fast toward its target—military or otherwise. What are the implications of intercepting or destroying that missile in the upper atmosphere?

Part of the answer to that question depends on knowing what conditions would allow lethal amounts of the liquid agent to reach the ground.

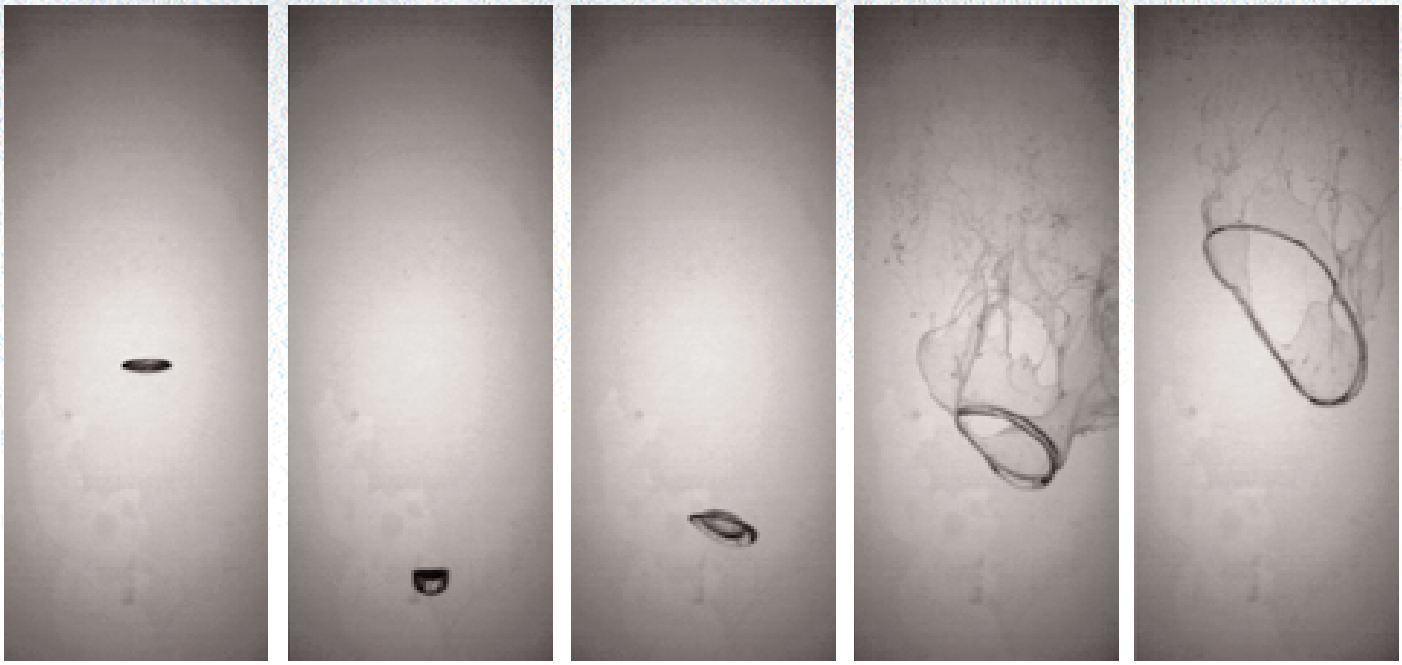
For instance, consider the chemical nerve agent VX, an organophosphorous compound that disrupts the body's nervous system. Lethal doses—ingested, inhaled, or absorbed through the skin—cause rapid death. It is estimated that a lethal dose is contained in a 2- to 3-millimeter-size drop. A warhead holding 400 kilograms of VX contains about 62 million lethal doses. If the warhead were to reach its target—say, a port or air base—it would saturate the target and cause an "area denial," that is, make the target site unusable until cleaned up. But what if it were to be intercepted tens of kilometers above the ground? What would happen to the VX?

The extreme conditions experienced by a single liquid drop during its reentry into the atmosphere lie in a regime for which no experimental data exist. To better understand the physics of what happens at these altitudes, physicist Glen Nakafuji, analyst Roxana Greenman, professor Theo Theafanous of the University of California (UC) at Santa Barbara, and research colleagues are studying how liquid breaks up and evolves in rarefied (thin) atmospheres.

To do so, they are using unique hydrodynamic and shock-physics experiments coupled with advanced chemical–kinetic and hydrodynamics computer codes. The experiments and codes simulate the supersonic, rarefied flow environments that reentering droplets of a chemical agent would experience. Nakafuji is the principal investigator for the project, which is funded by the Laboratory Directed Research and Development (LDRD) Program.

Thin Atmospheres, High Velocities, Surface Tension

A number of complicated factors determine how a body of liquid breaks up and how the individual drops or streamers



Series of photos showing "bag breakup" of a liquid drop, in which the round drop deforms into a shape resembling a bowler hat.

break apart and shape and reshape themselves. The factors include the pressure of the surrounding atmosphere, the velocity at which the liquid is traveling, and the physical properties of the liquid. "At altitudes of tens of kilometers," explains Nakafuji, "the agent disperses and expands in an atmospheric pressure that can be ten thousand times less than that at sea level. Pieces of liquid float out, stretch, and tear in milliseconds, then fall in an expanding cloud into the atmosphere." From there, the mass of drops falls through the air, moving at supersonic velocities through increasing atmospheric pressure. "Originally," notes Nakafuji, "people in the field theorized that the liquid would aerosolize into droplets on the order of 10 micrometers in diameter and disperse. Initial experiments indicate that this may not be true." So the question remains open: Would a given liquid break up into these small-size droplets or not?

"There's a huge gap in experimental data for the behavior of liquids in this sort of environment," notes Nakafuji. "We know how various liquids break up at sea level, where the atmosphere is dense, and the air molecules—which can be represented as individual particles—are constantly bouncing off each other, pressing together, and acting more like a fluid than individual particles." However, higher up in the atmosphere, the molecules are fewer and more widely dispersed, acting more like individual particles at altitudes above 30 kilometers. "You add to this the fact that the liquid agent is not in free fall but is experiencing atmospheric drag, and the problem becomes very complex," notes Nakafuji. "Yet this is the situation we're faced with in examining the physics of droplet breakup."

Of Weber Numbers and Bag Breakups

The physics of a liquid drop breaking up has much to do with the nature of the fluid (its density and viscosity, for instance) and the forces acting upon it. The ratio of external aerodynamic force—which tends to pull the drop apart—to the liquid's surface tension—which tends to hold the drop together—is a dimensionless quantity called the Weber number. Drops of different Weber numbers break up in different ways. Drops with higher Weber numbers (above 100) tend to have more catastrophic breakup and result in smaller drops. At very high altitudes, where external aerodynamic forces are small, the Weber number remains relatively low, below 100. When the team conducted experiments on drops with a range of Weber numbers characteristic of high altitudes, interesting findings emerged. For instance, drops 3 to 4 millimeters in diameter tended to oscillate before breakup. For drops with Weber numbers between 12 and 100, the experimenters observed a phenomenon called "bag breakup," in which a round drop deforms into a shape resembling a bowler hat, with a flat rim and curved crown. As the drop falls, the bag portion, which corresponds to the crown of the hat, oscillates in and out. When the original drop disintegrates, large drops form from the rim, and smaller ones form from the bag. "This happens in tens of milliseconds—much slower than anyone expected," says Nakafuji. "Previously, it was observed that such bag breakup would occur in hundreds of microseconds to 1 millisecond, tops."

ALPHA Goes with the Flow

These experiments were conducted in the ALPHA facility, a one-of-a-kind experimental system designed and built by the

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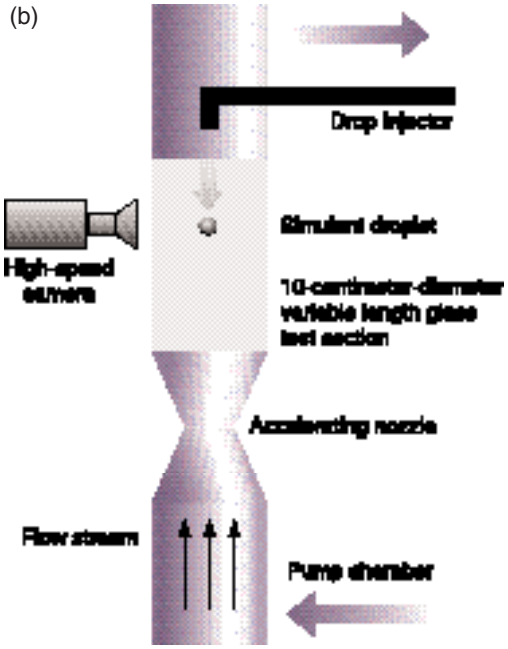
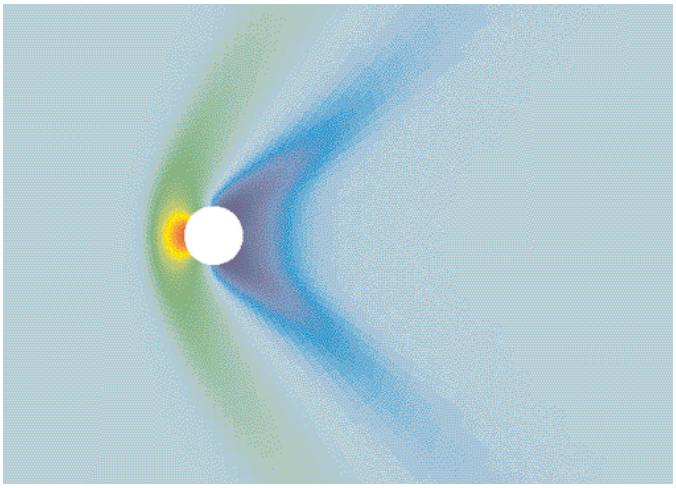
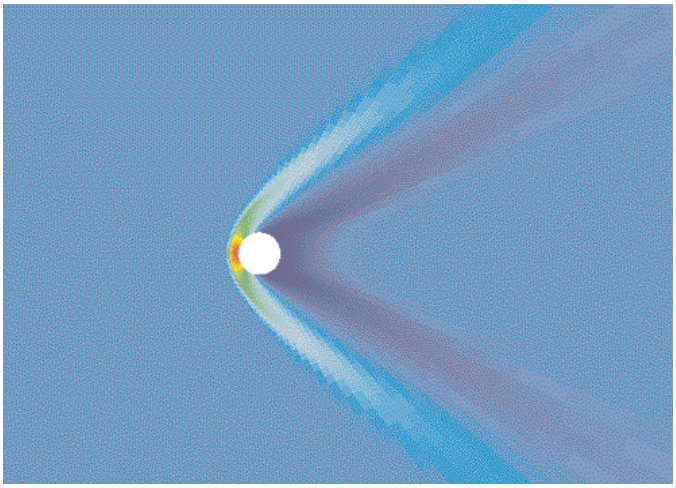


(a) The ALPHA facility is a one-of-a-kind experimental system to examine liquid fragmentation. (b) A diagram of the vertical wind tunnel used to re-create a drop falling through the upper layers of the atmosphere.

Livermore–UC Santa Barbara collaboration to examine liquid fragmentation. The facility is essentially a large, vertical wind tunnel, consisting of a cylinder about 3 meters long and 10 centimeters in diameter, that can be pumped down to pressures of 10 to 30,000 pascals. The methodology for re-creating a drop falling through the upper layers of the atmosphere is as follows. An injector releases liquid through a laser beam. The drop breaks the beam, which makes it act like an optical trigger and causes a diaphragm to burst. Air rushes up the cylinder past the drop, in effect simulating the fall of the drop through the atmosphere, and a high-speed camera records the behavior of the drop. "We have the capability to get air moving at velocities of Mach 5—about 1.5 kilometers per second," says Nakafuji. The air flows past the drop at a nearly constant velocity for about 200 milliseconds before its speed begins to ebb, long enough to watch a drop fall, reverse direction, rise, and then burst. This past spring, the group tested a drop 1.5 centimeters in diameter—the largest drop yet tested anywhere. "We don't test actual agents," Nakafuji emphasized. "We use glycerin and other kinds of fluid, and extrapolate to agents from there."

Besides examining whether assumptions made at sea level about the breakup of liquid hold true in rarefied environments, the team is also exploring the different break-up modes and whether the dynamics of these modes differ from the dynamics seen for bag breakup. The researchers' efforts





Simulations with Livermore's ALE3D code, which can predict the drag on rigid spheres in subsonic and supersonic rarefied flows, validate a surface-tension model, and test a deformable drop simulation.

have been rewarded. They have documented dynamics that have never before been seen or predicted. "For instance, before the bag breaks, it oscillates at some frequency," explains Nakafuji. "What we saw for the first time—and which no one had expected—is that after the drop turns and begins to move upward, the oscillation frequency doubles. We are now trying to understand this."

Getting Details, Drop by Drop

Ultimately, the team would like to understand and be able to predict the dynamics of specific liquid drops in any rarefied environment. "We'd like to be able to calculate the onset of breakup—when a drop will break up, the configuration the liquid will take, which drops are stable, and which are not," says Nakafuji, adding, "We've definitely made strides in that direction, to the point where we can now accurately predict whether a drop will break up under certain conditions."

The present goal is to obtain critical hydrodynamics and chemical data to validate computer models of these simulations. Working toward this end, the researchers have successfully used the Laboratory's ALE3D code to predict the drag on rigid spheres in subsonic and supersonic rarefied flows, validate a surface-tension model, and test a deformable drop simulation.

"Using experiments and simulations, we are pinpointing the ranges of drop stability and getting a better handle on the physics of liquid breakup," explains Nakafuji. "In the final analysis, we want to be able to predict the rarefied atmospheric conditions under which a given chemical agent will break up into lethal-sized stable droplets. This is a critical question, one whose answer could affect us all."

—Ann Parker

Key Words: ALE3D, ALPH facility, biological agent, chemical agent, lethality, liquid breakup, nerve agent, rarefied atmosphere.

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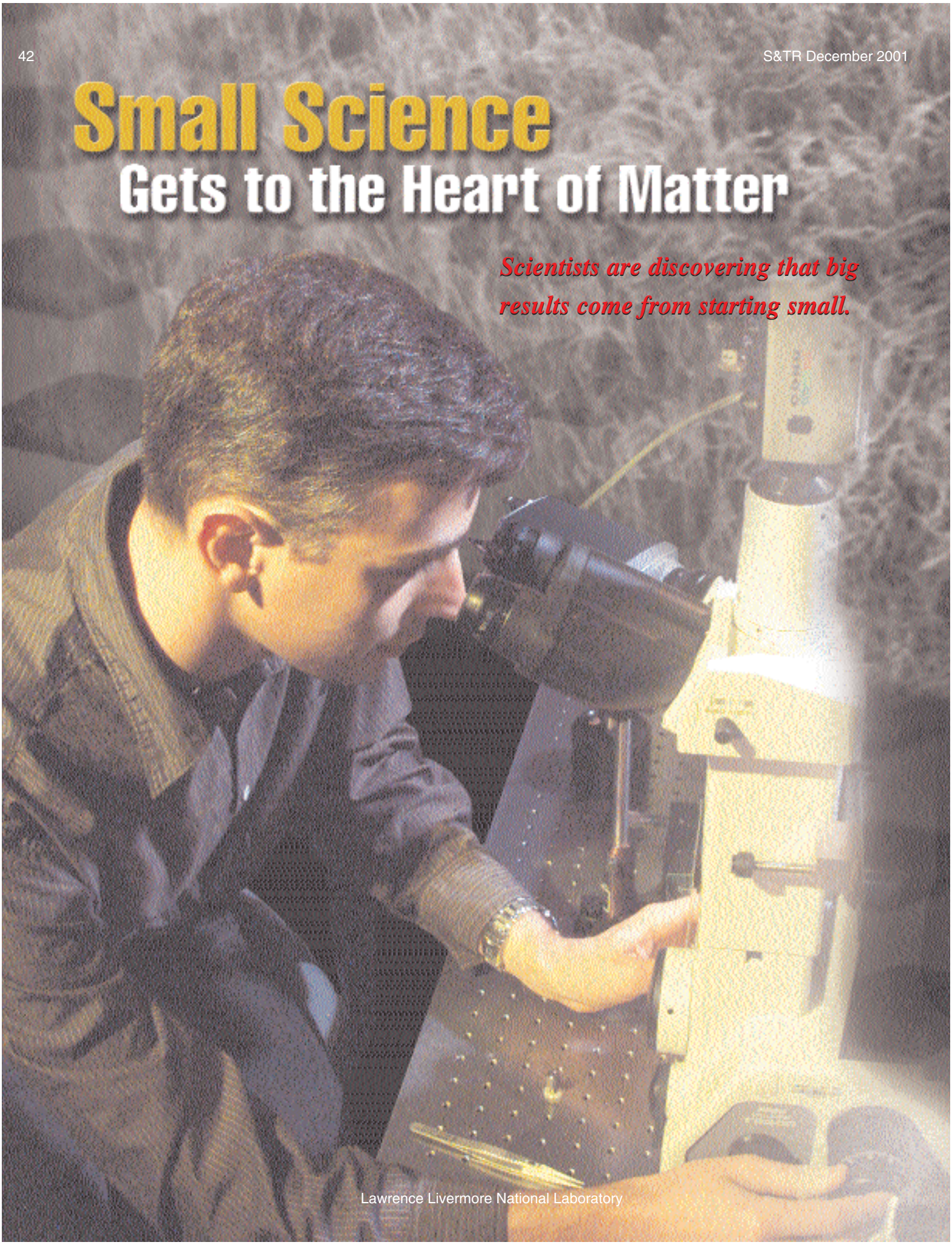
Small Science Gets to the Heart of Matter

Scientists are discovering that big results come from starting small.

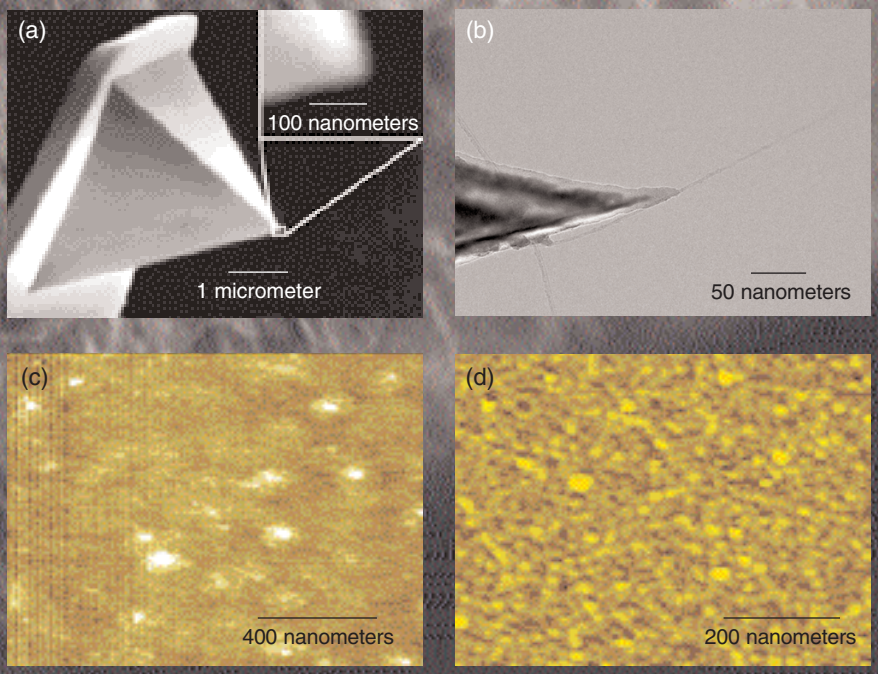


Lawrence Livermore National Laboratory

FINDING the best ways to detect biological warfare agents is one of Lawrence Livermore's missions today. Detecting large quantities of a biological pathogen is not difficult. The challenge is in detecting a few molecules of a toxin or a few bacteria or viruses to provide the early warnings of a biological attack.

Physicist Christine Orme and colleagues in the Chemistry and Materials Science Directorate are helping to understand some of the fundamental issues that underlie biodetection as well as fulfilling other Laboratory goals. They are performing research at minute scales in a field known as nanoscience, which takes its name from nanometer, a billionth of a meter. The team is examining, on an atom-by-atom and molecule-by-molecule basis, the organization of materials on surfaces and learning how that organization affects material properties. "One of the keys to working in nanoscience is controlling the surface and then being able to detect what is there," says Orme.

At the nanoscale, experimental results can be viewed only with the most powerful imaging tools. The atomic force microscope (AFM) has been used since the mid 1980s to produce topographic maps of nanostructures. Today, Orme's colleagues are developing new microscopic techniques based on use of the AFM that give even higher resolution and supply more than just topographic data. They are also refining the spectroscopic techniques that identify chemical bonds and supply fingerprints of molecules.



(a) Typical atomic force microscopy (AFM) tip and (b) nanotube tip. With the smaller nanotube tip, it is possible to obtain much more detailed information about a surface. AFM images of titanium grains obtained using (c) a typical AFM tip and (d) a nanotube tip.

The current research builds on pioneering Livermore work in crystal growth and thin multilayers, both of which depend on a keen understanding of material behavior at the atomic level. Livermore has a long-standing effort in crystal growth and characterization, born out of the need for large, ultrapure crystals in Livermore's lasers. Multilayers—exceedingly thin alternating layers of materials—were first demonstrated more than 50 years ago. But improved fabrication technologies developed by Livermore's Troy Barbee have prompted their use as highly reflective mirrors for telescopes as well as in a variety of optical applications, including electron microprobes, scanning electron microscopes, and particle beamlines in accelerators. (See *S&TR*, December 1999, pp.11–13.)

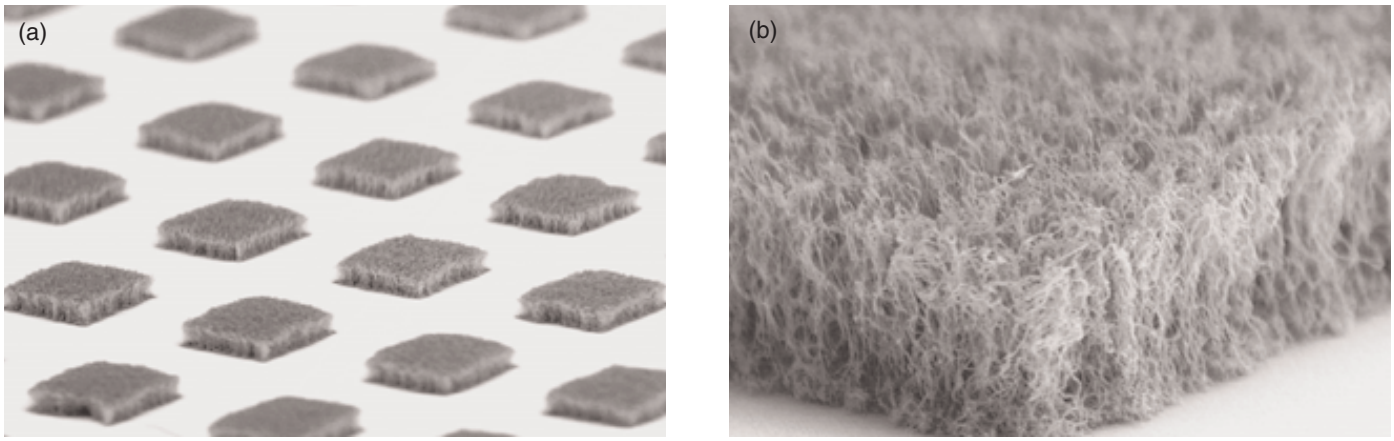
Seeing Is Believing

In atomic force microscopy, an extremely sharp tip senses the atomic shape of a sample while a computer records the path of the tip and slowly builds up a three-dimensional image. The AFM tip is positioned at the end of an extremely thin cantilever beam and touches the sample with a force of only 1/10 millionth of a gram, too weak to budge even one atom. As the tip is repelled by or attracted to the sample surface, the cantilever beam deflects. By imaging a larger or smaller area, researchers can vary the level of magnification of an AFM image.

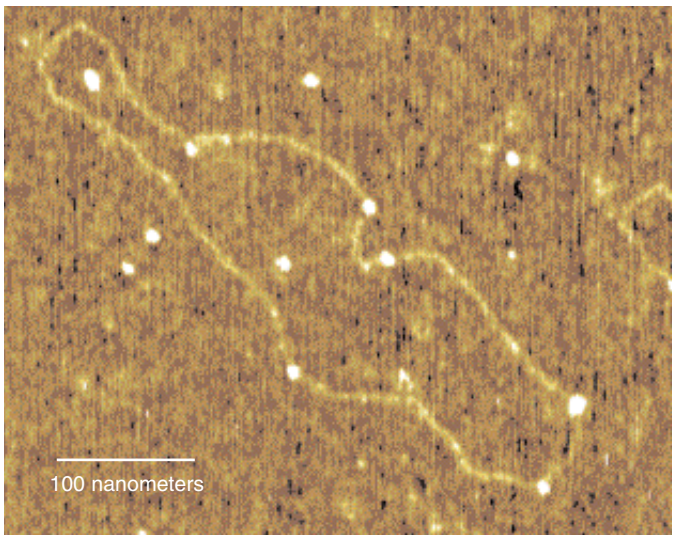
The AFM can also be adapted to sense a range of forces including attractive or repulsive interatomic forces, electrostatic forces, and magnetic forces.

But even the sharp tip of the AFM is sometimes not tiny enough for the small scale at which the research team is working. Physical chemist Aleksandr Noy is growing carbon nanotubes that can be used to replace the standard AFM tip. The figure above compares a typical AFM tip and a carbon nanotube tip. Carbon nanotubes are built of carbon hexagons that are arrayed in a configuration resembling chicken wire. They are 1/50,000th of the width of a human hair but a hundred times stronger than steel at one-sixth the weight. Noy can make many kinds of nanotubes—single wall, multiwall, thick, thin, single isolated, or large arrays. The smaller, lighter nanotube tip tracks the shape of an object more accurately to provide more detailed information about its surface.

Noy used the nanotube-tipped AFM to image the cucumber mosaic virus and reveal its structure fairly clearly. AFM images contain less information than structures revealed through x-ray diffraction techniques, but Noy's image was captured in minutes, whereas the same structure took over a year to resolve from diffraction data. "In principle, this technology could be used to image a single virus," says Noy. "Emergency workers could compare its



(a) "Farms" of carbon nanotubes and (b) a closeup of one farm. Livermore is exploring the potential of such nanotube arrays for detection applications.



One of the first images of DNA repair proteins bound to DNA.

Aleksandr Noy with the atomic force–confocal optical microscope.

image with a computerized database of known virus structures to identify it very quickly."

With the nanotube tip on the AFM, a team led by Noy also obtained the first unambiguous visualization of a DNA repair protein bound to DNA. By incorporating a synthetic mutagenic molecule into DNA and tagging a repair protein with a fluorochrome, they will be able to study the repair process in situ.

Another imaging technique being used by physicist Thomas Huser and others is confocal microscopy. It is based on a fluorescence microscope augmented with a pinhole that limits the volume being probed to get rid of extraneous background "noise." Its beam can be focused to 500 nanometers. The confocal microscope efficiently collects fluorescence emitted from fluorescent molecules that have been excited by laser light. With this spectroscopic technique, Huser has been able to detect single molecules.

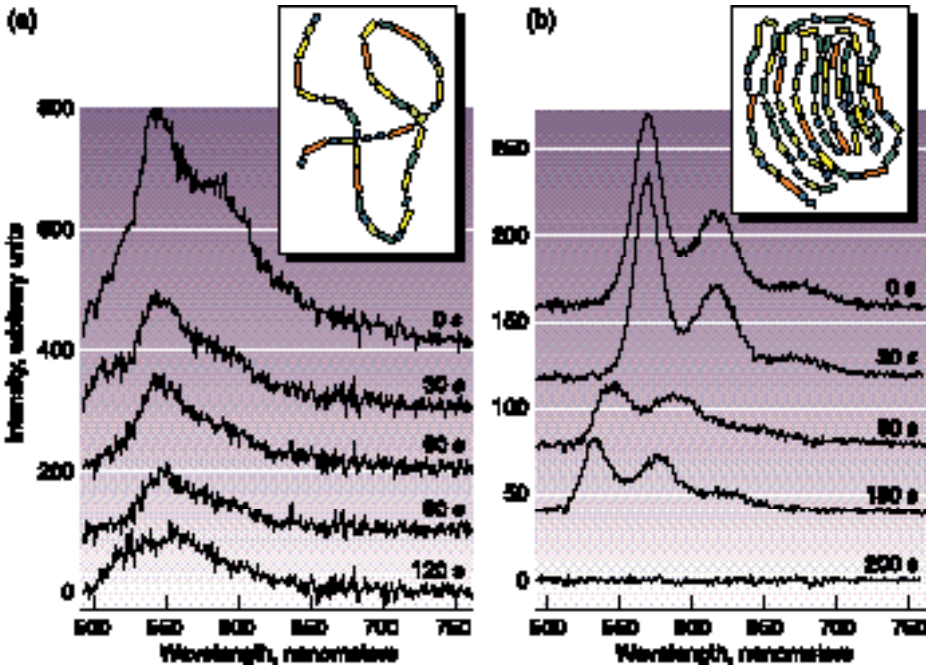
The confocal microscope is ideal for studying conjugated polymers, a new material that may be used to fabricate the next generation of light-emitting diodes (LEDs). Known as 2-methoxy, 5-(2′-ethyl-hexyloxy)-p-phenylene-vinylene, or MEH-PPV, the polymers are composed of a chain of benzene rings that emit light when linked by electrodes to which voltage is applied. The advantages of these polymers over the inorganic semiconducting materials of today's LEDs are many: They are easier to process on a large scale, they can be used to create ultrathin and flexible devices, and their power consumption is lower. Last year's Nobel Prize in Chemistry was awarded for the development of conjugated polymers.

Huser has learned that the physical configuration of the MEH-PPV molecules affects their fluorescence. "The photoluminescence of conjugated polymers depends strongly on how they are shaped," says Huser. When they fold up into a well-organized pattern in toluene, their shape enhances efficient energy transfer within the molecule. As conjugated polymers begin to be used as LEDs in electronics, some LED applications will take advantage of the high-energy-transfer configuration while others will benefit from the less ordered pattern for low energy transfer.

In experiments, Huser exposed MEH-PPV to two solvents, toluene and chloroform. In toluene, the MEH-PPV molecules curl up tightly because, says Huser, "they don't like toluene. They try to avoid it." Spectrographic data collected every 5 seconds show a slight flicker as the molecules die off with exposure to oxygen and the light they emit shifts from red to blue. In chloroform, the polymer spreads out. There is no blue shift, the light spectrum is broader, and the light intensity simply decays slowly with time.

Huser recently began experiments with the confocal microscope to examine the dynamics of single molecules of DNA. Fluorescent labeling of DNA, RNA, enzymes, and proteins is common laboratory practice to illuminate the interactions and functions of these important biomolecules.

At the same time, Noy has built a whole new microscope system that combines the topographic capabilities of the AFM and the spectroscopy of the confocal microscope. He will be



The development of photoluminescence over time in the conjugated polymer MEH-PPV, a material with multiple fluorophor segments on a chain. (a) MEH-PPV exposed to chloroform forms an open, irregular coil (see inset) that leads to luminescence from multiple sites, hence the broad spectral emission. (b) MEH-PPV exposed to toluene forms a tight coil (see inset) with strong overlap between segments. In this conformation, only the segments with the lowest transition energy emit light. Thus, the emission is narrow and more structured. Once all the red fluorophors are photodestructed, the segments with the next lowest energy begin to emit light at slightly blue-shifted wavelengths.

using this system to obtain even better information about DNA repair as well as new information on how DNA is packaged.

Identifying a Single Molecule

Another tool for identifying molecular species is Raman spectroscopy, a form of light scattering similar to fluorescence. Although Raman-scattered light is much less intense than fluorescence, the technique is a powerful analytical tool because the changes in wavelength of the weakly scattered light are characteristic of the scattering material. Raman spectroscopy can identify chemical bonds and obtain the unique fingerprint of a molecule. Every molecule has a unique Raman spectrum, but not every molecule fluoresces. Raman spectroscopy is one of the few optical techniques that can identify a molecular species and determine its chemical bonding by observing its distinct molecular vibrational frequencies.

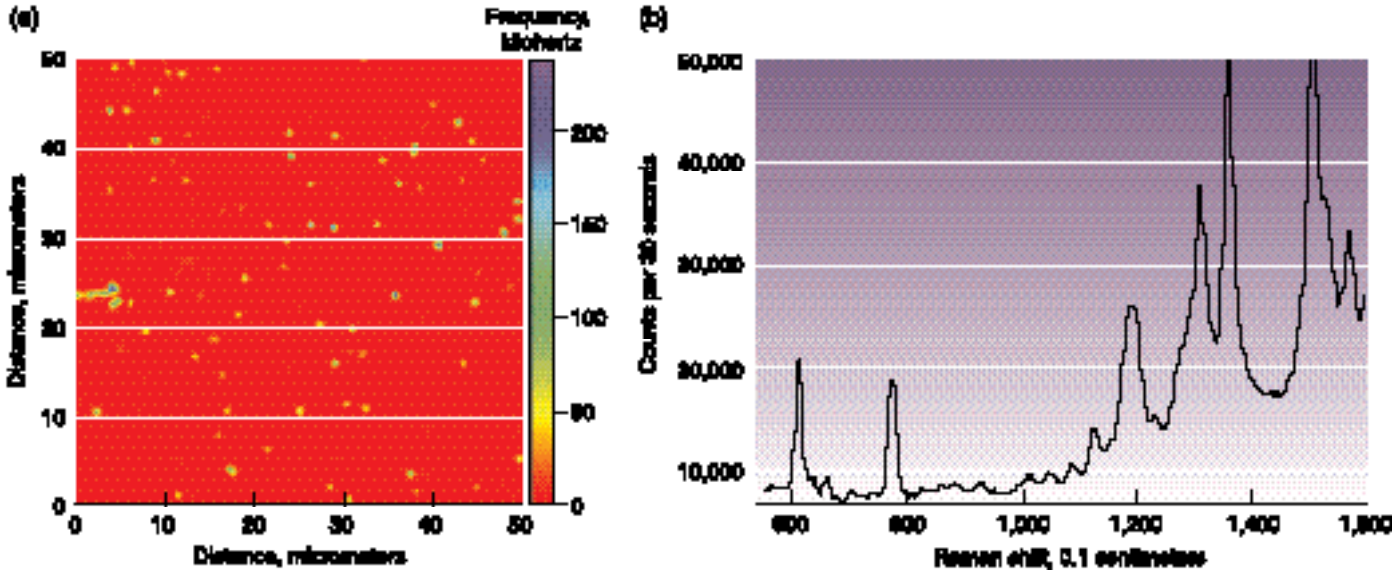
To increase the brightness and thus the resolution of Raman-scattered light, Huser has introduced nanometer-size gold crystals to the tip of a scanning probe microscope in a technique known as surface-enhanced Raman spectroscopy. The gold is negatively charged and attracts positively charged materials such as amino acids to adhere to kinks in the crystals. Electron density waves radiate from the corners of the gold crystals and increase the Raman signal by a factor of a quadrillion. At the same time, the scanning probe produces an image of the physical structure of the sample. The combined data allow for identification of single molecules. Unlike fluorescence, which fades with exposure to oxygen, the increased energy from the gold particles persists.

"Being able to characterize materials and chemical bonds at the level of a single molecule is a whole new capability for Livermore," says Huser. It is possible to perform Raman spectroscopy on single DNA molecules or proteins and to look for differences between individual cells. Using this technique, scientists also can detect and identify the byproducts or precursors of chemical agents such as the nerve gas sarin. This capability is important in the development of sensors for chemical warfare agents.

Controlling Biomolecules

Some nanoscience projects require the careful design of surfaces to collect and organize atoms, molecules, nanocrystals, colloids, cells, and spores. These surfaces are known as templates or, as Noy describes them, "landing pads" for toxins, proteins, and other biomolecules.

Livermore is exploring several techniques for creating templates. Physicist Jim De Yoreo is developing one method based on dip-pen nanolithography, which dips the tip of the AFM into an "inkwell" of organic molecules to "write" on an inorganic surface. As the tip moves across the surface, it makes a pattern that has almost no topographic relief but exhibits chemical contrast with the surrounding region. It is even possible to create multiple ink patterns with this method. The feature size is con-



An example of the benefit of surface-enhanced Raman spectroscopy. (a) Confocal optical micrograph of 60-nanometer-diameter gold nanocrystals loaded with just a few molecules of the laser dye rhodamine 6G. (b) Surface-enhanced Raman spectrum of one of the gold particles in (a) easily identifies the adsorbed rhodamine by its characteristic Raman signature.

trolled by such factors as tip coverage, humidity, and contact time with the substrate, or, in the case of lines, tip speed across the substrate. Examples of patterns created using a gold-coated mica surface for the substrate and 16-mercaptohexadecanoic acid for the ink are shown in the figure at right. This method has been used to deposit patterns of antibodies that would attract toxins and viruses, a first step in the development of nanostructured biosensors.

Another major area of research at Livermore's Biology and Biotechnology Research Program (BBRP) and elsewhere is in proteomics, the study of proteins. Cells produce particular proteins either all the time or as needed to prompt gene expression, that is, to turn a specific part of the genetic code on or off. Without proteins, our DNA could not operate properly. One of the best ways to examine the structure of a protein is to crystallize it and then subject it to x rays to obtain its unique diffraction pattern. During the crystallization process, molecules come together and separate (in a process known as nucleation) until a critical size is reached. Reaching that critical size can take a long time, and sometimes it does not happen at all. One goal of current proteomics work is to speed up the nucleation process and make it more likely that proteins will crystallize.

Dip-pen lithography, using a chemical that would prompt protein nucleation, is an option. "But," says Orme, "the size scale is a challenge. Proteins are extremely small, typically from 1 to 10 nanometers."

"If we make the pen's lines smaller, they won't be visible," adds Noy. So he and researchers in BBRP are developing a fluorescent ink for drawing lines with the density of a single molecule. In initial tests, a single-molecule line of the human chorionic gonadotropin (HCG) antibody has been successfully drawn. The next step will be to attract the HCG protein.

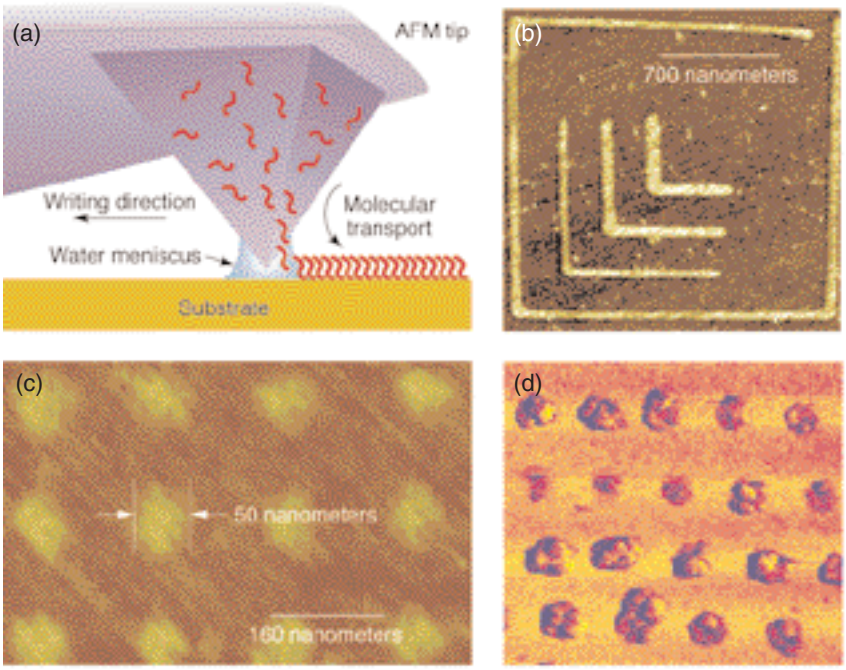
Nanolaminates, the next generation of multilayers, are also being explored as a way to accelerate the nucleation and growth of ordered proteins. Nanolaminate structures have been successfully synthesized with layers that are the same small size as typical proteins. The alternating layers have different surface charges, which prompt the proteins to adsorb in ordered rows. In the example shown in the top figure on p. 18, a nanolaminate was dipped into a solution of the protein ATCase. The nanolaminate was then removed, rinsed, air-dried, and imaged with AFM using a carbon nanotube tip. The resulting extremely high resolution of the image makes nonspherical proteins individually distinguishable on silica stripes. An image of the same deposition onto a homogeneous silica surface is very different, lacking any linear order. This set of experiments was the first step in accelerating nucleation and growing protein crystals that are suitable for x-ray diffraction.

Mimicking Natural Growth

Nanoscience is finding another application in the hands of Orme, De Yoreo, and colleagues whose research on the growth of calcite crystals sheds new light on the formation of bones, eggshells, and seashells.

The natural growth of organic crystals is known as biomineralization. Biomimetics is the term for mimicking nature's building methods to make a synthetic material. "We can only learn to make better bones and teeth if we first understand how the materials grow and interact with biological molecules," says Orme. "While there is a big step between this fundamental research and synthesizing materials that are truly similar to the real thing, we are part of the process to create better materials that affect health."

Pure calcium carbonate in the mineral form called calcite grows only in a symmetrical, six-sided rhombohedral-shape

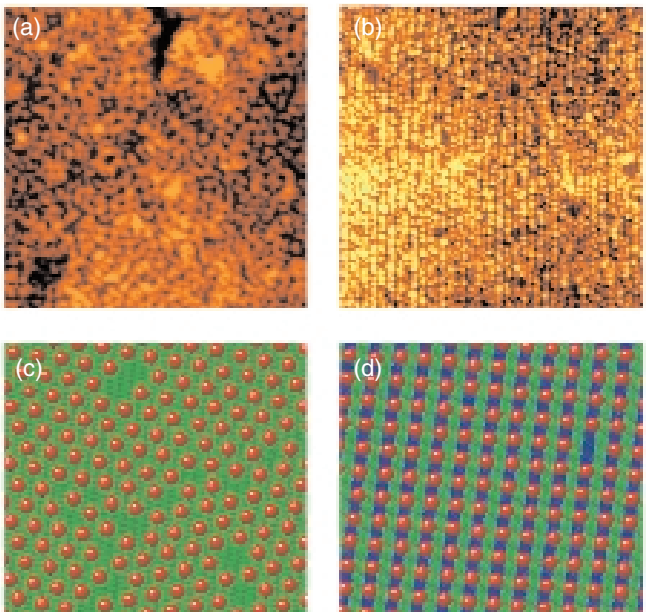


(a) Schematic of dip-pen nanolithography technique. Friction force images of (b) logos, (c) dots drawn on gold, and (d) colloid particles adsorbed preferentially on the dots. Features are composed of 16-mercaptohexadecanoic acid. The lines are 40 to 50 nanometers wide.

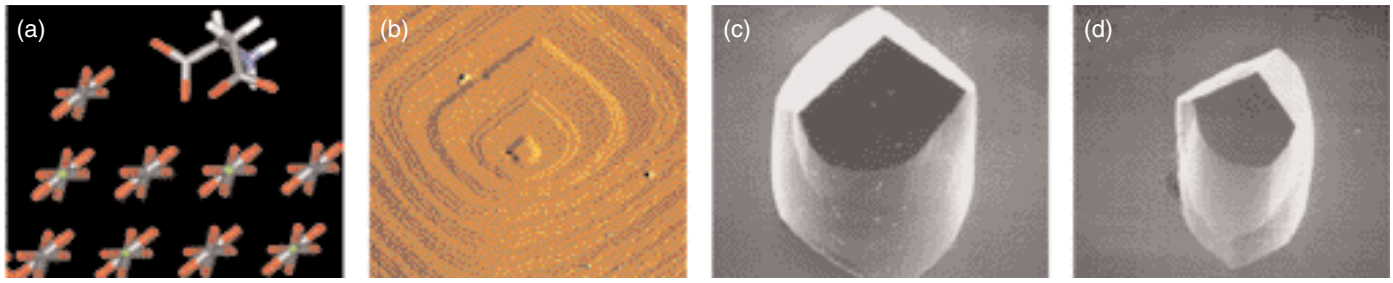
crystal. But that does not explain the intricate shapes found in nature, such as that of seashells. Researchers have known for a long time that organic molecules can influence the shape of a growing mineral crystal by attaching themselves to it. But it took experiments at Livermore to demonstrate the process in detail, showing how amino acids work at the molecular level to change a growing crystal.

In the experiments, the team added aspartate, one of the more abundant amino acids found in the proteins of shellfish, to calcite crystals growing in solution. Aspartate is typical of many amino acids in that it exhibits handedness, or chirality. As the researchers monitored crystal development, they found that the left-handed and right-handed form of the molecule attached more strongly to opposite atomic steps. The results were crystals that were mirror images of one another. The figure below illustrates how a chiral amino acid influences a growing calcite crystal. By knowing which steps the amino acid interacted with and using the symmetry relations of the crystal and the amino acids, the team was able to predict the binding position of the amino acid to the calcium carbonate step.

Comparable experiments are just beginning on calcium phosphate, the material used by animals to grow bones. Ultimately, experimental results may be put to myriad uses, from potential laboratory growth of human and animal bones to prevention of scale formation in pipes to the manufacture of toothpaste—any situation in which calcium-based crystals grow naturally or are used.



(a) A homogeneous silica substrate and (b) a nanolaminate of alumina and silica were dipped into a solution of the protein ATCase. Models show that (c) the deposition on the silica surface lacks any linear order, but (d) proteins adsorb to the nanolaminate in ordered rows, indicating the likelihood of growing ordered protien crystals suitable for x-ray diffraction.



The interaction of D-aspartic acid (D-Asp) with a calcite mineral surface. (a) Model illustrating the binding of Asp to a calcite step. (b) An atomic force microscope image of calcite steps (0.32 nanometers high) in a solution containing D-Asp. The steps of pure calcite are rhombohedral, but when an Asp-bearing solution is flowed into the fluid cell, the two lower steps interact with Asp and become curved. L-Asp binds more strongly to the left step, and D-Asp binds more strongly to the right step. These differences were used to deduce the binding motif. (c) An electron microscopy image of an approximately 10-micrometer-diameter calcite crystal nucleated on micropatterned, self-assembled monolayers in the presence of D-Asp. The atomic step structure in (b) is reflected in each of the three caps. (d) Crystals nucleated in the presence of L-Asp are mirror images of those nucleated with D-Asp.

Fundamental Science at Work

A nanostructured device is also finding its way into tests for the Yucca Mountain project, the nation's candidate for a repository for long-term storage of nuclear wastes. Tests of corrosion-resistant materials are being developed that use patterns formed by "writing" with voltage rather than with chemical inks. A voltage is applied between the AFM tip and a metal or semiconductor substrate to grow oxide patterns under the tip. In the figure below, an oxide greeting is written into a titanium film. The dot on the "i" is made larger and broader by applying a higher voltage. If the nanopatterns blur or dissolve during testing, the change provides a very sensitive indicator that the protective oxide film is changing.

This project is typical of so much fundamental research performed at Livermore. Using funding from the Laboratory Directed Research and Development (LDRD) Program, the oxide templates were originally developed to nucleate calcium phosphate minerals and to control protein deposition onto medical implants. Now, the Yucca Mountain project is putting the template to practical use. Much of the other work at Livermore to grow and image nanostructures also started as basic research, funded either by LDRD or by the Department of Energy's Office of Basic Energy Sciences, before finding a range of applications—including sensors that may someday be a lifesaver.

—Katie Walter

Key Words: atomic force microscope (AFM), biological sensors, biomineralization, carbon nanotubes, chemical sensors, confocal microscope, genomics, nanolaminates, proteomics, surface-enhanced Raman spectroscopy.

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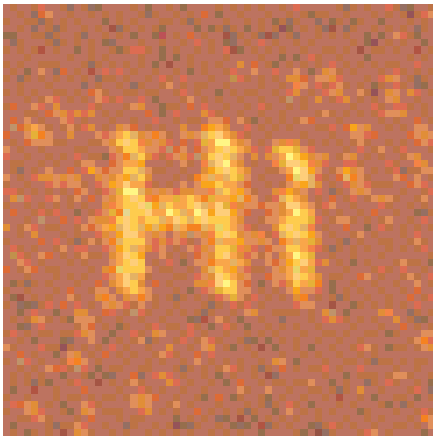


Atomic force microscope image (0.7 micrometers by 0.7 micrometers) of oxide grown on titanium using a voltage applied between the tip of the atomic force microscope and the substrate. (Image made by Livermore summer student researchers R. Sivamani and E. Bochner.)

About the Scientist

CHRISTINE ORME, a physicist in the Materials Science and Technology Division of the Chemistry and Materials Science Directorate, received a B.S. in physics from the University of California at Berkeley. She joined the Laboratory as a postdoctoral fellow after receiving her Ph.D. in physics from the University of Michigan in 1995. Her background is in experimental physics in the area of surface evolution and pattern formation during the growth of thin films. In her thesis work, she combined imaging with kinetic Monte Carlo simulations and continuum modeling to deduce diffusional processes during vapor growth. At Livermore, she uses this background to study crystal growth from solution (rather than from vapor). She is particularly interested in the area of biomineralization where organic molecules substantially change the shape of inorganic crystals; she wants to understand the formation of materials such as shells, bones, and teeth. Recently, she has become interested in the use of electrochemical driving forces to control electrodeposition and corrosive processes, particularly in their application to biomedical implants and corrosion-resistant industrial materials.

Simulation-Aided Design of Microfluidic Devices

Computer simulations help microfluidic device designers get from concept to prototype quickly and efficiently.

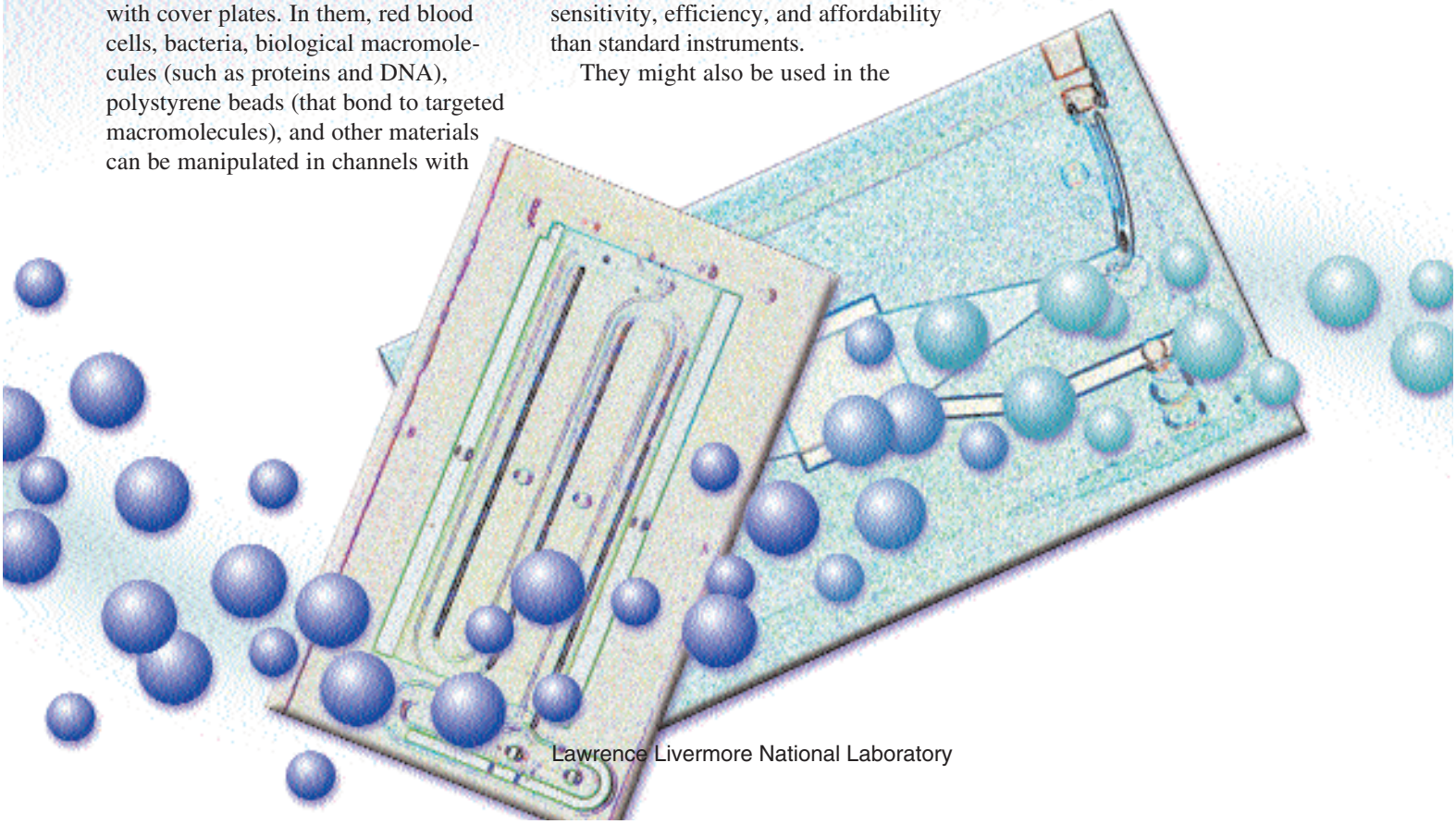
THE microchip revolution made possible today's miniaturized electronics industry. In like manner, the microchip is changing laboratory instruments that analyze fluids. Large and costly instruments are being replaced by microchip-based systems known as microfluidic devices. These miniature systems move fluids through a maze of microscopic channels and chambers that have been fabricated with the same lithographic techniques used for microelectronics.

Microfluidic devices are fashioned from silicon, glass, plastics, and ceramics into 2- or 3-square-centimeter slices with cover plates. In them, red blood cells, bacteria, biological macromolecules (such as proteins and DNA), polystyrene beads (that bond to targeted macromolecules), and other materials can be manipulated in channels with characteristic length scales on the order of 100 micrometers. The devices integrate sensors, actuators, and other electromechanical components to dispense with myriad moving parts and the people required to operate and service them.

Microscale instruments and processing are the future of medical research and the chemical and pharmaceutical industries. Microfluidic devices hold the promise of a small analytical laboratory on a chip to identify, separate, and purify cells, biomolecules, toxins, and other materials. They would perform these tasks with greater speed, sensitivity, efficiency, and affordability than standard instruments.

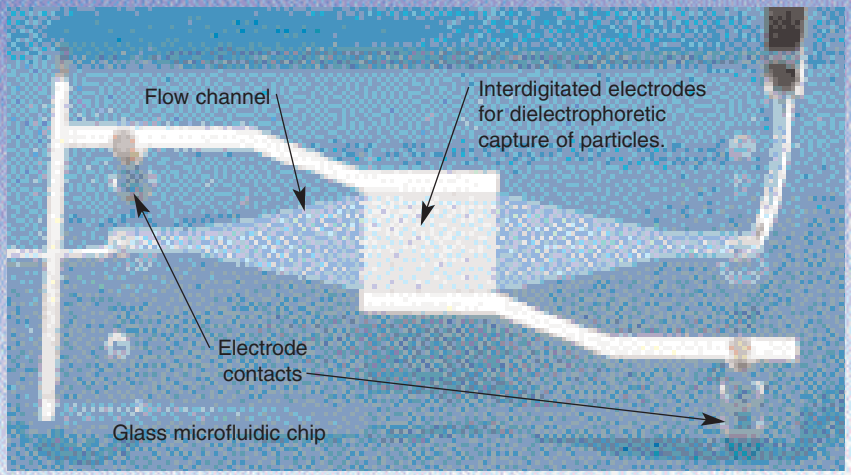
They might also be used in the future for detecting chemical and biological warfare agents, delivering precise amounts of prescription drugs, keeping tabs on blood parameters for hospital patients, and monitoring air and water quality.

For more than a decade, Lawrence Livermore researchers have been working on several aspects of microfluidic devices. The Laboratory's Center for Microtechnology has more than 30 experts in electronics, biology, optics, and engineering who are developing microfluidic components for transporting, sensing, separating,



Lawrence Livermore National Laboratory

mixing, and storing fluids and their constituents. (See *S&TR*, July/August 1997, pp. 3–9.) Current Livermore projects include the design and prototyping of devices for the human genome program, chemical and biological warfare agent detection, and medical analysis.



In actual size, this microfluidic device designed by Livermore engineer Peter Krulevitch is barely larger than a postage stamp.

First Complete Model Designed

To help guide the design of microfluidic devices at the Center for Microtechnology and elsewhere, a team of Livermore researchers is developing a complex, three-dimensional simulation tool. The team consists of chemical engineers David Clague and Elizabeth Wheeler, postdoctoral mechanical engineer Todd Weisgraber, and University of California (UC) at Berkeley student Gary Hon. In this work, they collaborate with other Livermore researchers from several disciplines as well as colleagues at universities. The team has been funded for the past three years by the Laboratory Directed Research and Development (LDRD) Program through Livermore's Center for Computational Engineering and, more recently, by the Defense Advanced Research Projects Agency (DARPA) of the Department of Defense.

The team's computer code has drawn increasing interest because it provides an accurate representation of the behavior of suspended particles, especially polystyrene beads and biological macromolecules, as they travel inside a microfluidic device. The simulation capability incorporates into a single numerical code complex channel geometries and such parameters as fluid flow rates, particle interactions, and external forces. "We want to predict the complex interplay of the forces involved in microfluids to give designers a way to accurately predict how beads, cells, and macromolecules will behave," says team leader Clague.

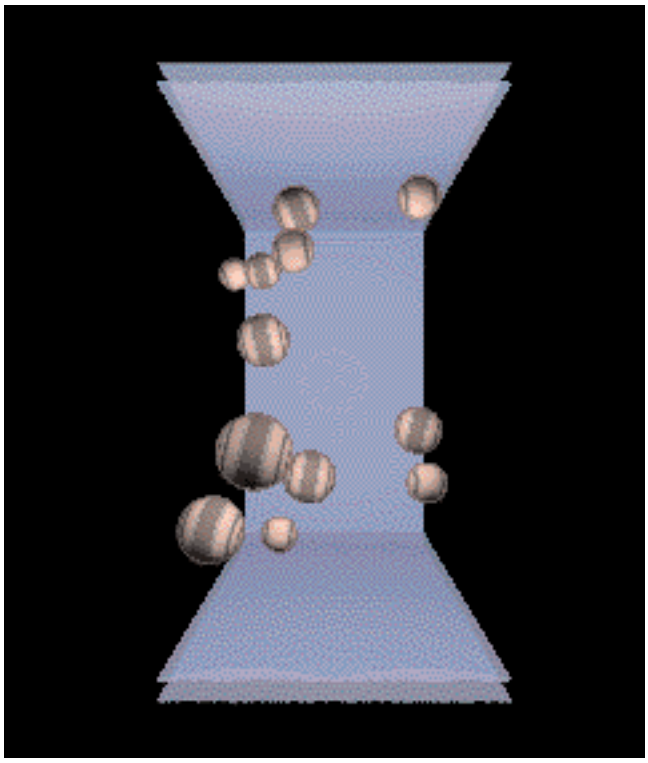
Clague notes that suspended particles traveling within microscopic channels are subject to a number of physical forces that influence their transport and separation from each other and the channel walls. The forces, such as subtle electrical attractions and repulsions, can be used to achieve the movement and manipulation of suspended particles in ways that would not work in traditional bench-scale laboratory instruments.

The Livermore simulation capability provides a new tool to assist microfluidic device designers who

want to engineer systems that will reliably move, separate, concentrate, and identify suspended particles of interest. With effective simulation, the designers can see the effects of design decisions before they build a prototype. For example, a designer may want to position selected biological macromolecules in the central region of a microchannel for capture by an electric field and therefore must determine what field strength will be required. Or a designer may want to see how restricting a channel with a tiny post might affect the fluid flow rate and the mixing behavior of particles as they are forced to "slalom" around it.

The program uses a form of the Boltzmann transport equation called the lattice Boltzmann equation (LBE) to represent the behavior of fluids and suspended particles within microfluidic devices. (Ludwig Boltzmann was an Austrian physicist whose greatest achievement was the development of statistical mechanics, which explains how the microscopic constituents of matter—atoms and their properties—determine macroscopic properties such as thermal conductivity or viscosity.) In recent years, the LBE method has gained popularity and usefulness in simulating the flow of complex gases and liquids. It is based on a statistical description of the fluid on a cubic lattice in which each lattice site represents up to several thousand individual fluid molecules.

In the team's numerical model, spheres represent polystyrene beads and biological macromolecules within the lattice. The spheres can be assigned different sizes, densities, and electrical properties. Because of their size, the spheres can occupy several lattice sites. The code tracks the spheres as they move on the lattice and calculates the extent to which the spheres interact with each other, the channel walls, the fluid, and external forces that may be applied to manipulate them. The simulation tracks the time evolution of both the fluid and suspended spheres. The algorithms (mathematical routines) used by the program tend to be readily applied, allowing calculations in a straightforward manner and making it easy to incorporate new forces.



Simulations can accurately reflect a host of physical forces that act on suspended particles flowing in a microfluidic device that typically measures 100 micrometers long, wide, and high. These forces, such as subtle electrical attractions and repulsions, are typically of much less importance in traditional bench-scale laboratory instruments.

A Natural for Parallel Computing

Because the LBE method is naturally suited for parallel computing, the simulation capability is designed for large computers, preferably supercomputers that use tens to hundreds of microprocessors together. Simulations representing time scales on the order of tens of seconds of continuous suspension require a few days of computer time. The team uses several Livermore machines for their simulations, including the Compass Cluster and two massively parallel supercomputers: Blue, the 740-gigaops unclassified portion of Blue Pacific, one of the Department of Energy's Accelerated Strategic Computing Initiative supercomputers, and the 680-gigaops TeraCluster2000. (See *S&TR*, October 2001, pp. 4–10.) The TeraCluster2000 is the preferred computing platform; simulations on it use up to 50 microprocessors working simultaneously.

One important advantage of the code is its flexibility. The simulated suspended particles can be assigned different physical and electrical attributes, including electrostatic forces that cause fluids containing biological macromolecules to act far less predictably than ideal species, which would consist of hard, inert spheres. External forces such as gravity, alternating current, or direct current can be simulated. These forces can be turned on and off to isolate their

specific effects on particle behavior. Livermore engineer Peter Krulevitch, a microfluidic device project leader, says that until now, no program was capable of simulating all the forces acting on fluids containing particles. "The problem has just been too complex," he says.

The LBE method contrasts with traditional fluid modeling based on finite-element analysis and boundary element methods, which typically deal with pure fluids. Results from the Livermore code, however, can be handed off to larger-scale computer-aided design simulation tools that use standard finite-element analysis.

Mike Pocha, a Center for Microtechnology section leader, notes that device designers can build prototype devices—a long and painstaking process—and determine their capabilities or, preferably, simulate them first and then build a prototype guided by the simulation results. Going from concept to manufacturing a prototype is increasingly more time-consuming and expensive as microfluidic devices get more complex, says Clague. "With a more comprehensive simulation tool, researchers will be better able to predict what will happen to the suspended species in these complex microenvironments. Ultimately, such a capability will speed the design effort and reduce costs."

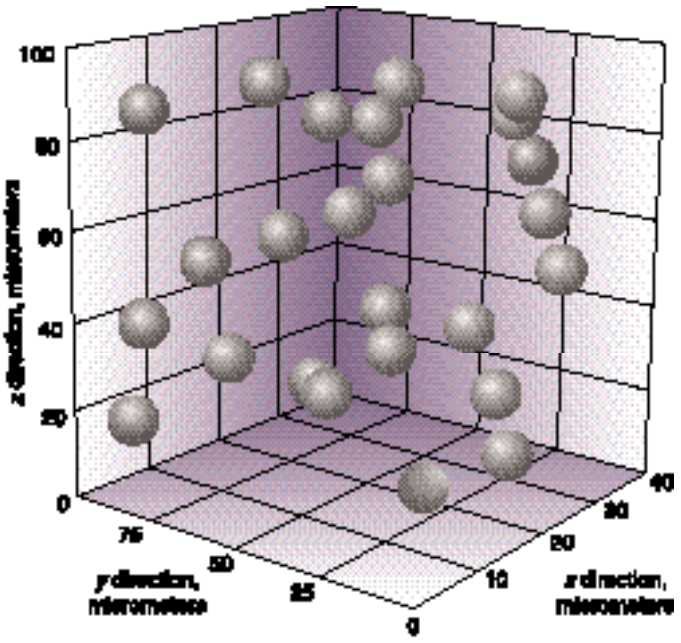
The physics involved with the operation of microfluidic devices is complex and varies, depending on the fluid, the molecules suspended in the fluid, and the extent, if any, of external fields. In building the code, the team has steadily added capabilities that more completely represent the physical forces at work in microfluidic devices. After every addition of a new feature, the team makes sure the results are in excellent agreement with existing theory and, where possible, with published alternative numerical methods.

LDRD Laid the Groundwork

One of the team's first accomplishments under LDRD funding was simulating hydrodynamic forces acting on a stationary sphere. These forces are dependent on the velocity of the suspending fluid and the proximity of the suspended particles to channel walls. The LBE method naturally takes into account the entire spectrum of fluid and particle behavior, including inertial effects and hydrodynamic interactions between suspended particles. In other words, the simulations account for the minute disturbances propagated within a fluid by the particles that "feel" each other's presence and, as a result, change their trajectories and the properties of the fluid.

The hydrodynamic forces, including inertial effects, are particularly well captured. The first is the drag force, which is a result of the fluid exerting a force on a suspended particle because of differences in fluid and particle velocities. The second force is a lift force, which is caused by small inertial effects and gradients in fluid velocity. The lift force is exerted perpendicular to the flow, causing the species to migrate to the center of the channel. Also coming into play is a particle's density, which affects its buoyancy within a fluid and the extent to which it can be lifted.

Fluids normally flow through microfluidic channels without turbulence so that suspended particles typically mix only by diffusion. One of the key parameters used to characterize fluid



Simulations using the lattice Boltzmann equation method are based on a cubic lattice, here with dimensions of 40 by 100 by 100 micrometers. Spheres (in this example, measuring 5 micrometers in diameter) represent polystyrene beads and biological macromolecules within the lattice. The simulations track the spheres as they move on the lattice and calculate the extent to which they interact with each other, the channel walls, the fluid, and the external forces that are used to manipulate them.

flow is the Reynolds number, which defines flow conditions and measures the relative importance of inertial effects to viscous effects. Most fluid flow in small channels occurs at a low (but finite) Reynolds number. However, even at small Reynolds numbers, researchers have found that there are small lift effects. The Livermore simulation capability takes into account these inertial effects for predicting the extent of lift as a function of Reynolds numbers.

The code also simulates the effects on particles that are near channel walls. Much like the effect of a boat's wake, the motions of molecules cause disturbances in the fluid that bounce off the channel walls and reflect back on the particles. Close to the walls, particles experience forces retarding their motion, and even closer to the walls, they experience large resistive forces known as lubricating forces.

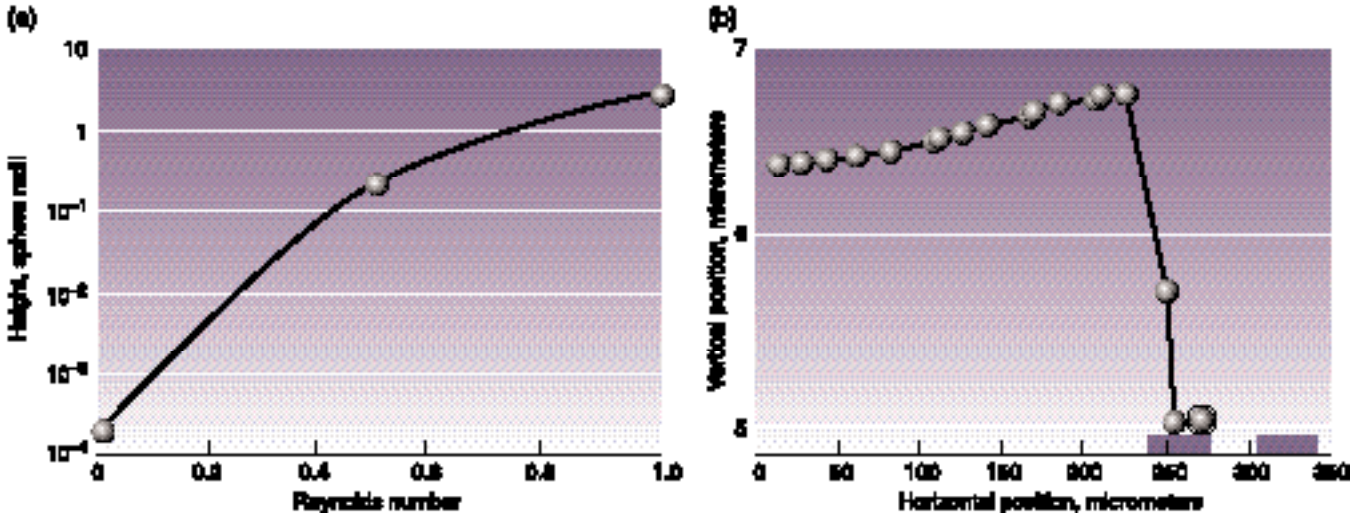
The Livermore simulation work is part of the Simbiosys (Simulation of Biomolecular Microsystems) program administered by the Defense Advanced Research Projects Agency. The program funds the development of advanced computational tools for the BioFluidic Chips design effort.

Adding Real Effects

If the simulation is to be accurate, it must also account for non-Newtonian characteristics that are exhibited by biofluids containing human cells, bacteria, and biological macromolecules such as proteins and DNA. These materials do not behave like electrically neutral and perfectly round spheres. Instead, they have widely varying shapes, densities, and often electrical charges that are asymmetrically distributed.

More importantly, these materials tend to have elastic character, which gives rise to unexpected effects. Strands of DNA, for example, can be long and gangly with a preferred, three-dimensional shape that orients itself in a particular manner to its neighbors. If forced to travel through a narrow channel, the strands deform but then exert a small force in an attempt to recover their favored configuration, much like a compressed spring reverts to its normal shape. If there is a sufficient concentration of such strands, this restoring force can have a profound effect on fluid behavior.

Depending on their concentration, particles interact with each other and with the channel walls. Under certain conditions, they can coagulate with each other or stick to walls because of van der Waals and electrostatic forces (electrical attraction and repulsion forces between species). The simula-



(a) The simulation capability can be used to predict the extent of inertial lift as a function of the fluid's Reynolds number. The lift force acts to push suspended particles up or down toward the center of the channel. (b) Dielectrophoresis (DEP) is an efficient method for capturing selected particles in microfluidic devices. DEP electrodes (rectangles) generate nonuniform alternating current electric fields that induce electrical polarization in biological macromolecules. The DEP forces overcome inertial lift forces to cause a selected particle to move toward the electrodes and to remain there.

tion team is incorporating these and other forces associated with biological macromolecules into the models, including hydrophobic (water hating) and hydrophilic (water loving) interactions. Clague explains that some proteins have hydrophobic regions that cause the proteins to aggregate when they are in close proximity to other proteins; therefore, these unique forces must be taken into account.

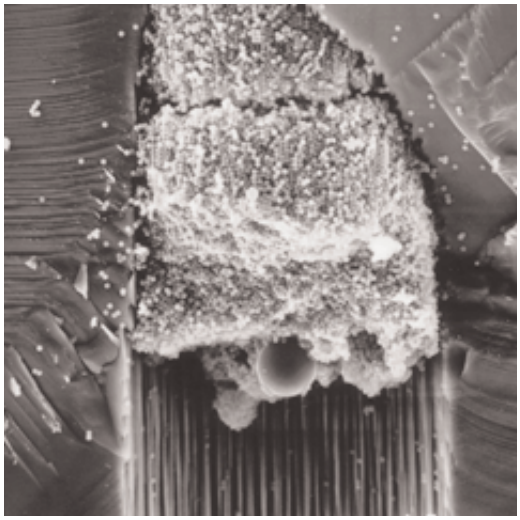
Last August, the team began work for DARPA, the advanced research arm of the Department of Defense and a major backer of microfluidic technology. One of DARPA's goals is to develop devices called BioFluidic Chips (BioFlips) that will identify biological macromolecules and microbes based on certain electrical or chemical properties. Soldiers would use BioFlips devices both to detect chemical and biological agents and to monitor their own general health. (See the box on p. 10.) As part of the microfluidic development effort, a program called Simulation of Biomolecular Microsystems (Simbiosys) is funding the development of advanced computational tools for the BioFlips design effort. The Livermore team's simulation work is part of the Simbiosys program.

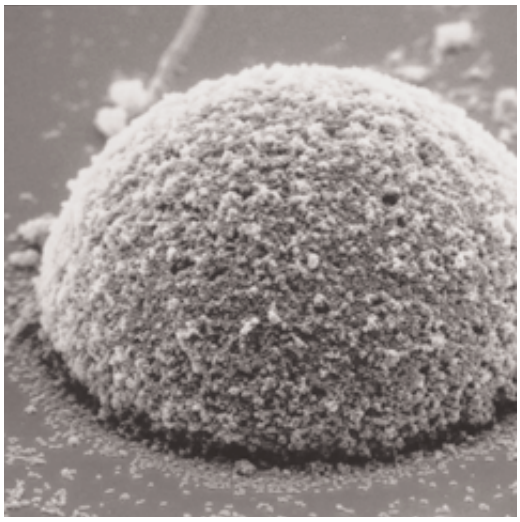
Focus on Dielectrophoresis

The team's work for DARPA builds upon LDRD research, particularly with regard to simulating the coupling of hydrodynamic and dielectrophoretic forces. Dielectrophoresis (DEP) is an efficient and increasingly popular method for separating molecules in microflows. DEP electrodes generate nonuniform, alternating current electric fields that induce electrical polarization in target species. On an absolute scale, the force is quite small, but in microfluids, the force can be quite effective in manipulating and positioning biological macromolecules with electrodes using less than 10 volts. The degree of induced polarization is dependent on the electrical properties of the molecule, the surrounding fluid, and the magnitude and frequency of the applied electric field.

"Different species typically have their own unique dielectric response fingerprint that can be exploited by DEP," says Clague. As a result, DEP can be used to select from among a number of different particles suspended in the same fluid. The selected particle will either be drawn toward or repelled from the region of high field intensity (toward or away from the DEP electrode located within a channel wall). The first instance is referred to as positive DEP, and the second is referred to as negative DEP.

DEP forces can be switched on and off to selectively capture cells, bacteria, spores, polystyrene beads, DNA, proteins, and other matter. Once captured, the molecules can be held in place or, with the removal of the force, sent on their way to a different location for analysis. For example, DEP can be used to selectively capture a suspected pathogen. The pathogen would then be shuttled to a different area where its





The Laboratory team is collaborating with University of California researchers at Berkeley and Davis to simulate the transport of suspended particles in microneedles. These simulations are helping to obtain a better understanding of why particles can stick together and plug microneedles, as shown. (Photos courtesy of Professor Dorian Liepmann of the University of California at Berkeley.)

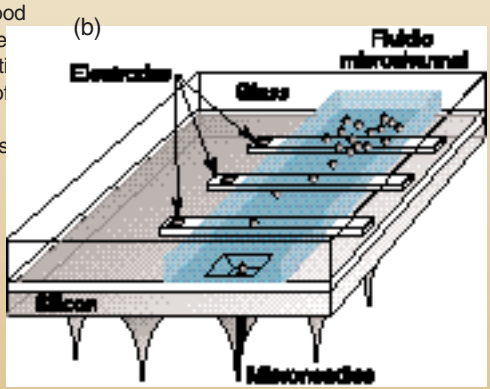
DNA would be extracted and analyzed.

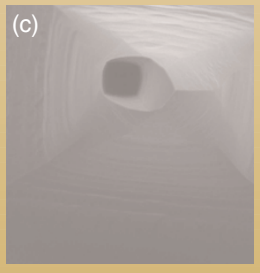
The DEP simulation work involves close collaboration with pathologist Peter Gascoyne at the University of Texas M.D. Anderson Cancer Center in Houston, Texas. Gascoyne and his colleagues, in a project sponsored by DARPA, are developing an instrument that uses DEP to separate cells and identify them based on their dielectric properties. A prototype has been used on whole blood samples to separate malignant cells from normal cells.

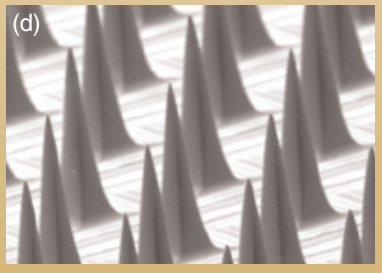
An important group of simulations is focused on examining the interplay of suspended particle concentration, flow rates (and inertial lift effects), and DEP forces with the effects from different kinds of suspended particles. Preliminary simulations show that the hydrodynamic interactions between particles can screen and thwart DEP forces; therefore, concentration effects become very important. The suspended particles that are not screened encounter a positive DEP force and are pulled to the electrode surface, where they are held motionless.

The team is continuing to enhance the numerical model to investigate the forces influencing DEP manipulation of molecules suspended in flowing fluids. One research avenue they are taking is to give biological macromolecules more realistic characteristics. For example, the team has explored replacing the simulated

Monitoring the Health of Soldiers







(a) The Defense Advanced Research Projects Agency is developing BioFluidic Chips (BioFlips) that are small enough to be worn on an earlobe and can identify biological macromolecules based on certain electrical or chemical properties. (b) A BioFlip uses an array of microneedles for continuous blood monitoring. (c) Vie of a microneedle ti and (d) an array o microneedles. (Photo and figures courtesy of Professor Rosemary Smith of the University of California at Davis.)

The BioFluidic Chips (BioFlips) program of the Defense Advanced Research Projects Agency (DARPA) is developing a clinical lab on a chip. BioFlips would offer all the advantages of microfluidic devices: miniaturized channels and reservoirs for increased speed of reaction, increased sensitivity, reduced cost of reagents, and reduced power consumption. The devices would be capable of rapid detection of infections and chemical and biological warfare agents, making possible potentially rapid treatment. BioFlips would be worn directly on the skin, perhaps on the earlobe for continuous blood monitoring through microneedles.

BioFlips would provide real-time, unobtrusive monitoring to directly assess the health of defense personnel. A commander could continuously monitor the status of troops—whether they are fatigued or have been exposed to biological threats, including bacteria, viruses, and toxins. The devices could monitor such entities as white blood cells, antibodies, blood pH, and blood glucose.

BioFlips promise fast health assessment, from seconds to minutes, in contrast to laboratory blood cultures using traditional methods that take hours or even days to process. If successful, the technology could perhaps be extended to improve national health care by unobtrusive and continuous monitoring of high-risk patients.

BioFlips designers need powerful computational tools to guide and speed their efforts. Hence, DARPA is sponsoring an allied DARPA program called Simulation of Biomolecular Microsystems (Simbiosys). The Simbiosys program recognizes that engineers have limited understanding of biological molecules and biochemical reactions and, furthermore, that biologists do not generally have knowledge about key biochemical reaction rates and little knowledge about the behavior of biological molecules in microscopic channels. The goal is the creation of what DARPA managers are terming the first "interface between biology and engineering." Effective simulation models will enable greater understanding of the transport of biological materials at the micrometer scale to enable better control and efficiency of the devices.

spheres with more accurate bead-and-spring representations of long-chain polymers such as DNA fragments. Also under development are representations of cell properties unique to organelles and membranes, which can significantly influence the response. Finally, the team is working on the inclusion of electrostatic and van der Waals forces as well as hydrophobic and hydrophilic interactions.

The team has collaborated with UC Berkeley researchers on developing arrays of 50-micrometer-diameter needles. The goal is to deliver drugs more efficiently, but interactions between particles cause the microneedles to become clogged. The Livermore team's simulation work is targeted at obtaining a better understanding of the problem. This work complements a DARPA-funded project at UC Davis, where researchers are developing microneedle arrays for drawing body fluids painlessly to monitor soldiers' health on the battlefield.

Clague expects the simulation program to become increasingly useful as applications for microfluidic devices expand. By providing a tool that allows microfluidic device designers to turn the variety of physical forces at play on and off, the team hopes to make possible the discovery of new ways to manipulate suspended particles. Such detailed and accurate simulations speed the design and development of novel microfluidic devices. As a result, the simulation effort may well have an important role in saving soldiers' lives and in developing new medical devices that could help drive down national health care costs.

—Arnie Heller

Key Words: BioFluidic Chips (BioFlips), Center for Microtechnology, Defense Advanced Research Projects Agency (DARPA), dielectrophoresis (DEP), lattice Boltzmann equation (LBE), microfluidic devices, Reynolds number, Simulation of Biomolecular Microsystems (Simbiosys).

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About the Scientist

DAVID CLAGUE is a staff engineer in the Electronics Engineering Technologies Division of the Engineering Directorate. He joined the Laboratory in 1998, after a year as a postdoctoral researcher at the Los Alamos National Laboratory Center for Nonlinear Studies. Clague received a B.S. in chemical engineering from the University of California at Santa Barbara in 1987, an M.S. in engineering in 1993, and a Ph.D. in chemical engineering in 1997, both from the University of California at Davis. His research specialties are in transport phenomena, complex fluids, microfluidics, and numerical methods. At Livermore and previously at Los Alamos, he has developed three-dimensional simulation methods for modeling particulate behavior. This work has been published in a number of refereed journals. Additionally, Clague has experience in industry, working for four years as a research and development engineer at Space Systems Loral to provide engineering and technical support related to polymeric composite materials and adhesives.

Zeroing In on Genes

ALMOST every cell in the human body contains the same set of genes. But not all of the genes are used, or expressed, by those cells. For example, some processes that are particular to cells in the liver are completely unused in brain cells. Ever since genomic research began, scientists have been searching the tangle of DNA for the expressed genes, the ones that really matter.

If one thinks of the nucleus of a cell as a library, then the chromosomes in the cell are bookshelves and the genes are the books on each shelf. Almost every cell in an organism contains the same libraries and the same sets of books. The books represent all of the information (the DNA) that every cell in the body needs so that it can grow and carry out its various functions. Two challenges complicate the process of locating our genes: Not all of the genes are expressed in any one tissue, and less than 10 percent of our DNA is actually used to make genes. Only occasional passages in the library's written material are important.

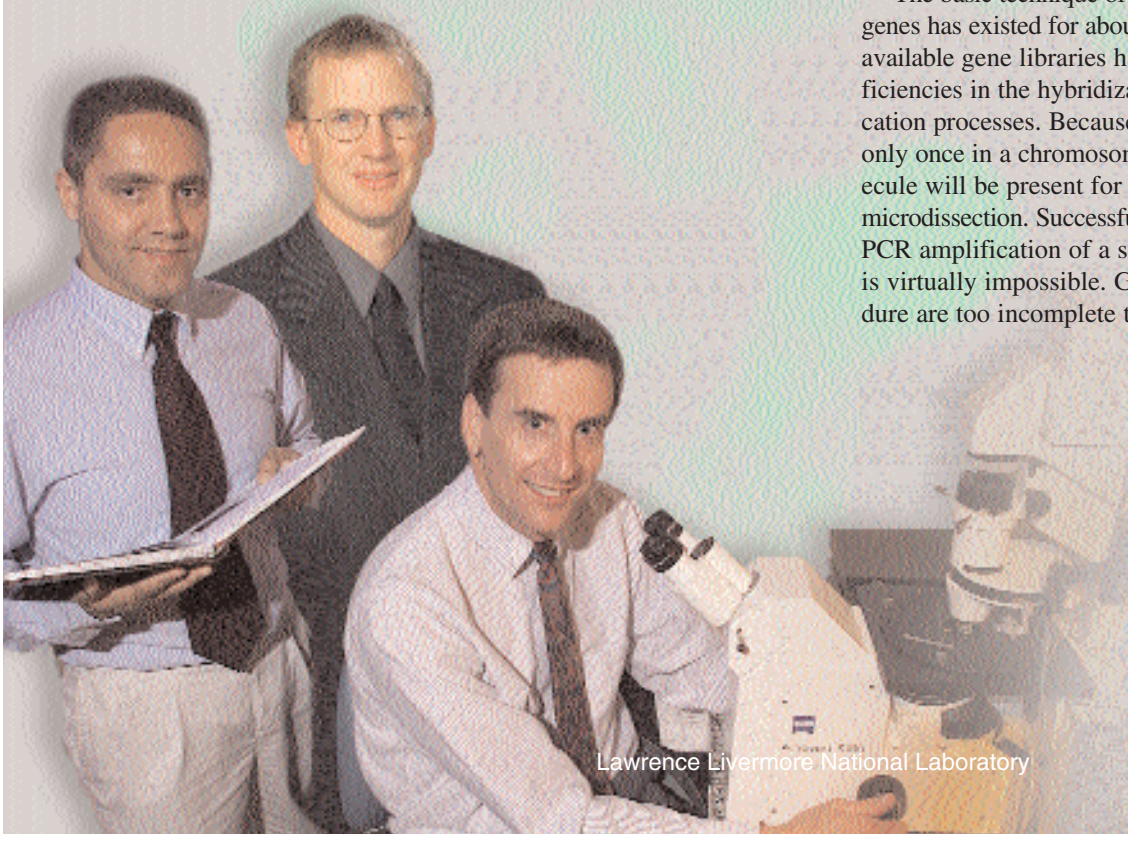
A team at Livermore led by molecular biologist Allen Christian has developed Gene Recovery Microdissection (GRM), a process that can weed out the unexpressed genetic material from a piece of DNA. With GRM, scientists can isolate all of the genes in a chromosomal region that are being used by a specific tissue at any point in time. GRM can be used for any plant or animal species. A variant of this method can also be used to clone all of the DNA of any organism, including bacteria, even those that cannot be cultured.

"It's not always necessary to sequence the entire genome of a species to locate its gene," says Christian. "With GRM, we can focus on particular regions of a genome that are of interest."

Amplification Twice Does the Trick

The product of gene expression is messenger RNA (ribonucleic acid), or mRNA. Typically, before work begins to isolate expressed genes, the mRNA molecules are converted into more stable complementary DNA molecules called cDNA, which has exactly the same sequence as the mRNA but is easier to handle in the laboratory. Then the cDNA is combined on a microscope slide with chromosomes. The cDNA molecules hybridize to the chromosome regions corresponding to the genes of which their parent mRNA is a product. Using tiny glass needles and microdissection, scientists can isolate regions of the chromosomes of interest and, with them, the hybridized cDNA molecules. Finally, amplification by polymerase chain reaction (PCR) is used to produce many copies of the molecules in preparation for DNA sequencing.

The basic technique of using microdissection to isolate genes has existed for about five years. But no commercially available gene libraries have been generated because of inefficiencies in the hybridization and subsequent PCR amplification processes. Because genes are typically represented only once in a chromosome, a maximum of one cDNA molecule will be present for each expressed gene following microdissection. Successful hybridization, dissection, and PCR amplification of a single molecule is virtually impossible. Gene libraries made with this procedure are too incomplete to be useful.



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Developers of the award-winning Gene Recovery Microdissection process are (left to right) Matthew Coleman, Allen Christian, and James Tucker of Livermore's Biology and Biotechnology Research Program Directorate.

Livermore's GRM process overcomes this inefficiency by combining cytogenetics and genomics with chromosome microdissection. CRM increases both the number of targets available for cDNA hybridization and the total number of cDNA molecules in each region following hybridization.

The trick is to perform PCR amplification in situ, on the slide rather than in a tube, which is the conventional means. And it occurs twice. First, prior to hybridization, random-primed PCR of the chromosomes on the slide produces many copies of the target DNA, significantly improving the chances of cDNA hybridization. Second, following the hybridization, another PCR amplification using primers specific for the ends of the cDNA molecules increases the numbers of bound cDNA molecules. Instead of isolating just one cDNA molecule per expressed gene in a region, the GRM process recovers hundreds or even thousands of cDNA molecules. This simple step makes possible the production of highly useful chromosome-region-specific libraries.

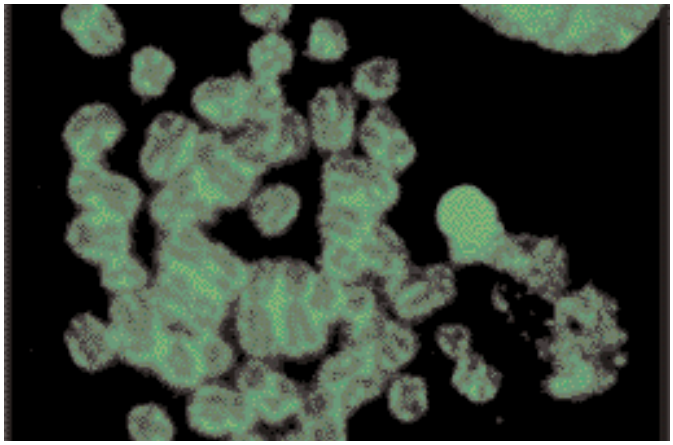
GRM has other advantages. While cells generally contain only one or two copies of a gene, some genes make thousands of copies of mRNA and others make only a few copies. Finding mRNA molecules with a low number of copies amid the "noise" of the more numerous gene products can be difficult with conventional methods of making cDNA libraries. But the hybridization step in GRM results in a balanced library in which mRNA molecules with high and low numbers of copies are equally represented.

Several companies offer processes that provide partial information about gene expression and genomic location. But no other single technique identifies both known and unknown expressed genes and determines the part of the genome that regulates their expression. GRM makes possible in one process what multiple processes could previously handle only in part, and it does so cost effectively. Current estimates are that the costs associated with GRM will be substantially less than those of traditional methods. The process is also significantly faster.

Benefits Abound

GRM was invented to allow researchers to identify cancer genes in chromosomal regions for which no genomic information existed. Initially, these were regions for which scientists had good evidence of their importance in rat mammary cancer but almost no other knowledge. To identify the genes expressed in these regions, researchers needed a quick, simple, inexpensive, and reliable method of identifying and characterizing both new and previously known genes in chromosomes.

GRM focuses on data that current genomic sequencing efforts do not provide, namely, information concerning the expression of genes in specific regions of abnormal cells, such as those found in cancerous tissue. "We are using GRM to learn which genes are expressed in certain parts of chromosomes in cancer cells," says Christian. "We can then compare our data with data from the Human Genome Project and learn how these particular cancer cells differ from normal cells."



Chromosomes amplified by Gene Recovery Microdissection. Amplification is by polymerase chain reaction and produces many copies of stable complementary DNA molecules in preparation for sequencing.

GRM will be used to generate chromosome-specific and chromosome-region-specific libraries of genes that are expressed for any tissue, normal or diseased, of any organism that can have its chromosomes spread on a microscope slide. Once these libraries have been produced, they can easily be placed on microarrays and made available to other investigators for more detailed analyses, including gene expression studies. GRM can thus be used to create a systematic approach to identifying genes expressed in virtually every species of interest to humans. This capability opens the door to sequencing many plant and animal species that might otherwise be ignored because of the prohibitive cost of genomic analysis. Agriculture, environmental sciences, and veterinary medicine will all benefit.

GRM technology provides the preliminary step toward a full genomic analysis of an organism, allowing time and money to be saved during the full analysis. This invention will enable scientists to identify genes that are expressed after exposure to drugs, environmental chemicals, or radiation. Toxicologists can study the reactions of cells and organisms to chemical and radiation exposure, furthering basic understanding of the molecular mechanisms involved in responses to adverse environments. Similarly, the pharmaceutical industry will be able to decipher biological responses to drugs.

—Katie Walter

Key Words: Gene Recovery Microdissection (GRM), genomic research, R&D 100 Award.

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Uncovering Bioterrorism

DNA-based signatures are needed to quickly and accurately identify biological warfare agents and their makers.

WITH the end of the Cold War, the threat of nuclear holocaust faded but another threat emerged—attack by terrorists or even nations using biological agents such as bacteria, viruses, biological toxins, and genetically altered organisms. The former Soviet Union once had a formidable biological weapons program. Now, several countries and extremist groups are believed to possess or to be developing biological weapons that could threaten urban populations, destroy livestock, and wipe out crops.

Even terrorists with limited skills and resources could make biological weapons without much difficulty, says Tony Carrano, Lawrence Livermore's associate director for Biology and Biotechnology Research. "It's not complex, it's not expensive, and you don't need a large facility." For these reasons, biological weapons have been dubbed the poor man's atomic bomb.

Contributing to the ease of making and concealing biological weapons is the dual-use nature of materials to pro-

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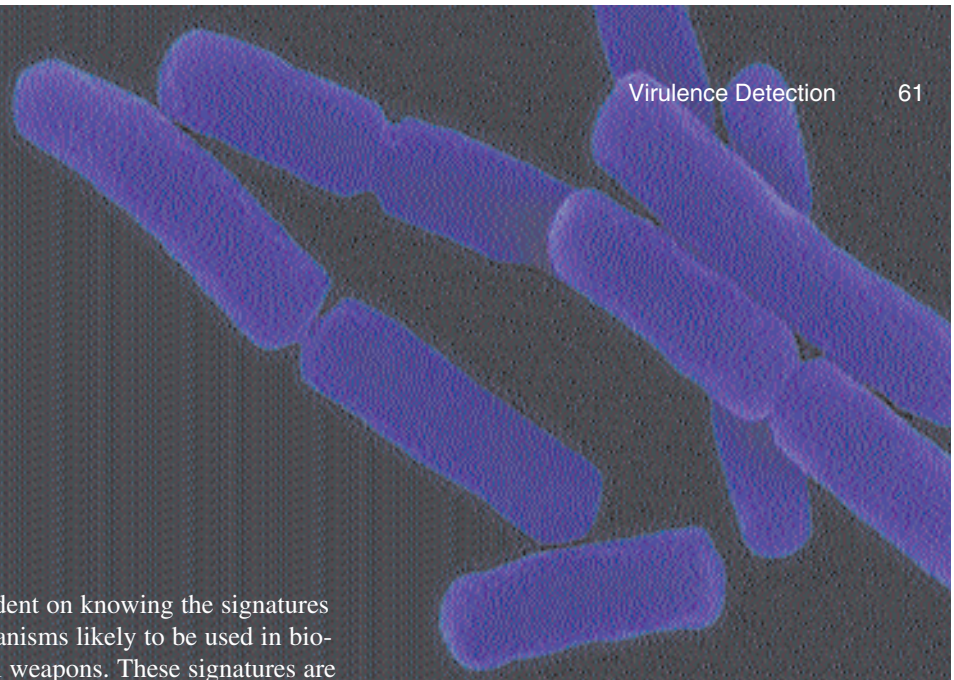
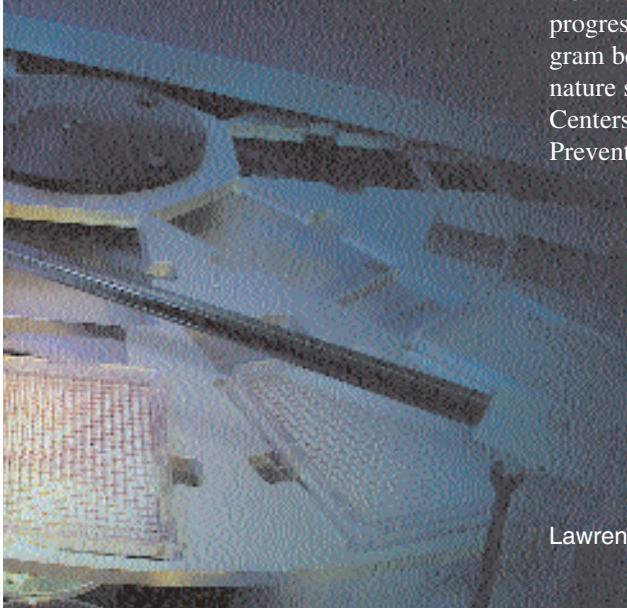


duce such weapons, because they are found in many legitimate medical research and agricultural activities as well. CIA Director George Tenet touched on this topic in Congressional testimony in February when he noted the overlap between manufacturing vaccines and producing biological weapons.

The agents used in biological weapons are difficult to detect and to identify quickly and reliably. Yet, early detection and identification are crucial for minimizing their potentially catastrophic human and economic cost. Lawrence Livermore scientists are participating in the Department of Energy's program to improve response capability to biological (as well as chemical) attacks on the civilian population.

A major part of DOE's program is developing better equipment, both fixed and portable, to detect biological agents (see *S&TR*, June 1998, pp. 4–11). However, any detection system is dependent on knowing the signatures of organisms likely to be used in biological weapons. These signatures are telltale bits of DNA unique to pathogens (disease-causing microbes). "Without proper signatures, medical authorities could lose hours or days trying to determine the cause of an outbreak, or they could be treating victims with ineffective antibiotics," says Lawrence Livermore's Bert Weinstein, deputy associate director of Biology and Biotechnology Research.

Because of the importance of biological signatures, DOE has launched a biological foundations program as a key thrust of its effort to improve response to terrorist attacks. The program involves experts at the Lawrence Livermore, Brookhaven, Los Alamos, and Sandia national laboratories, as well as colleges and universities. Researchers from the four national laboratories get together at least quarterly to share information and yearly for a formal review of their work. Weinstein reports that important progress has been made since the program began in early 1997, and new signature sets are being transferred to the Centers for Disease Control and Prevention and the DOE.



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Over the next several years, DOE scientific teams expect to produce species-level signatures for all of the most likely biological warfare pathogens. The teams also expect to have an initial set of species-level signatures for likely agricultural pathogens, because an attack on a nation's food supply could be just as disruptive as an attack on the civilian population.

Several Levels of Signatures

The teams also aim to develop strain-level signatures for the top suspected agents. Strains are a subset of a species, and their DNA may differ by about 0.1 percent within the species. A species, in turn, is a member of a larger related group (genus), and its DNA may differ by a percent or so from that of other members of the genus.

Characterizing pathogens at the strain level requires significantly more work than recognizing a species. But strain-level signatures are essential for determining the native origin of a pathogen associated with an outbreak; such information could help law enforcement identify the group or groups behind the attack.

The biological foundations work aims to provide validated signatures useful to

public health and law enforcement agencies as well as classified signatures for the national security community. In developing these signatures, biological foundation researchers are also shedding light on poorly understood aspects of biology, microbiology, and genetics, such as immunology, evolution, and virulence. Increased knowledge in these fields holds the promise of better medical treatments, including new kinds of vaccines.

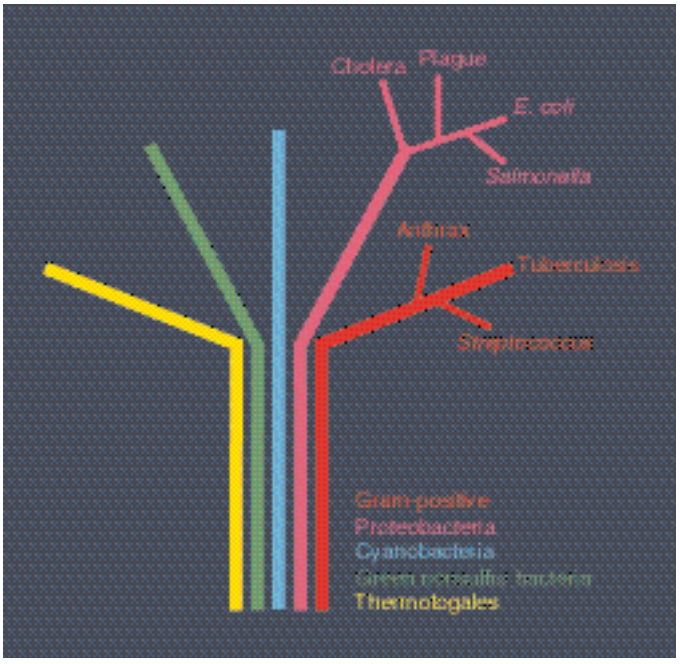
The biological foundations work is one element in DOE's Chemical and Biological Nonproliferation Program. Livermore's component of this work is managed by its Nonproliferation, Arms Control, and International Security Directorate. Other components of the overall program include detection, modeling and prediction, decontamination, and technology demonstration projects.

Livermore researchers were among the first to recognize, in the early 1990s, the tremendous potential of detectors based on DNA signatures. "We knew that a lot of work was necessary to develop the signatures the new detectors would need," says Weinstein. In particular, the researchers recognized several pitfalls. For example, if signatures are overly specific, they do not identify all strains of the pathogen and so can give a false-negative reading. On the other hand, if signatures are based on genes that are widely shared among many different bacteria, they can give a false-positive reading. As a result, signatures must be able, for example, to separate a nonpathogenic vaccine strain from an infectious one.

Several Levels of Identification

To enhance their detection development effort, researchers are exploring advanced methods that distinguish slight differences in DNA. They are using the multidisciplinary approach that characterizes Livermore research programs. In this case, DNA signature development involves a team of microbiologists, molecular biologists, biochemists, geneticists, and computer experts. In addition, the Livermore work benefits from collaborations with experts worldwide, extensive experience with DNA sequencing, and affiliation with DOE's Joint Genome Institute (see *S&TR*, April 2000, pp. 4–11).

Much of the work is focused on screening the two to five million bases that comprise a typical microbial genome to design unique DNA markers



This phylogenetic tree is a simple representation of the bacterial kingdom. All human bacterial pathogens belong to the Gram-positive (red) or Proteobacteria (magenta) divisions. The other divisions consist of nonpathogenic bacteria associated with diverse environments. Biological signatures must be able to differentiate infectious bacteria from hundreds of thousands of harmless ones. Each genus of bacteria has many species, and each species can have thousands of different strains.

DNA signature development involves a multidisciplinary team of microbiologists, molecular biologists, biochemists, geneticists, and computer experts. Here, biomedical scientists Peter Agron and Lyndsay Radnedge are performing suppressive subtractive hybridization to distinguish DNA of various species of virulent organisms.

that will identify the microbe. The markers, called primer pairs, typically contain about 30 base segments and bracket specific regions of DNA that are a few hundred bases long. The bracketed regions are replicated many thousands of times with a detector that uses polymerase chain reaction (PCR) technology. Then they are processed to unambiguously identify and characterize the organism of interest.

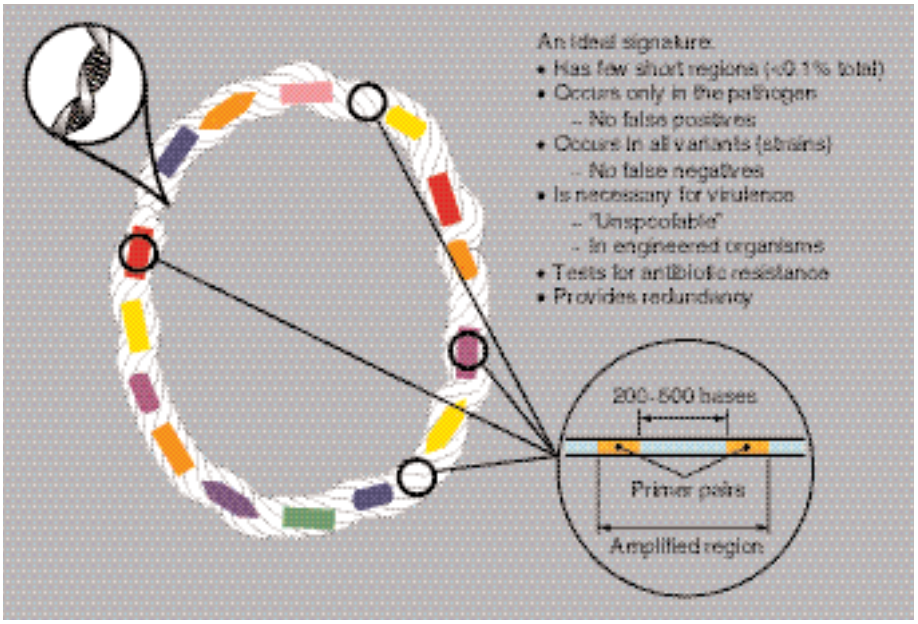
Weinstein notes that different signatures will be needed for different levels of resolution. For example, authorities trying to characterize an unknown material or respond to a suspected act of bioterrorism will begin with fairly simple signatures that flag potentially harmful pathogens within a few minutes. Typically, such a signature would encompass one or two primer pairs and be sufficient for identification at the genus level (*Yersinia* or *Bacillus*, for example) or below.

A signature in the next level of resolution is needed for unambiguously identifying a pathogen at the species level (*Yersinia pestis*, for example). This signature involves about 10 primer pairs. Currently, it takes several days to obtain conclusive data for a species-level signature. The goal is to reduce that time to less than 30 minutes.

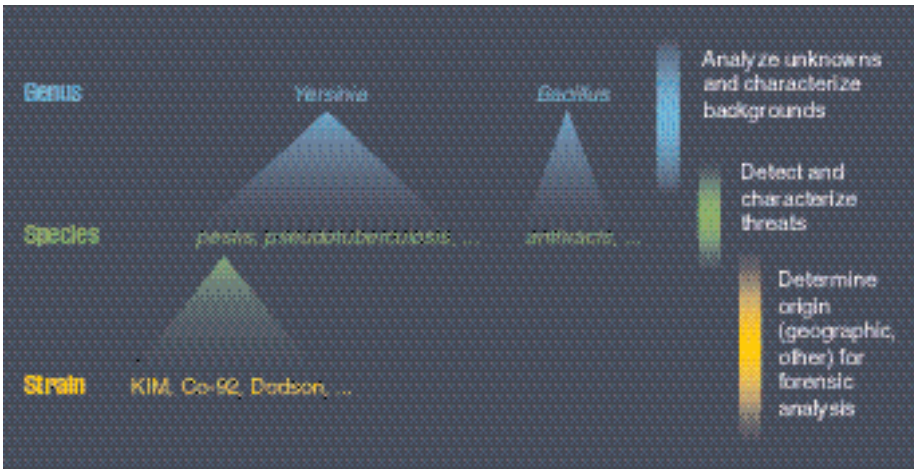
The third signature level is used in pathogen characterization, identifying any features that could affect medical response (for example, harmless vaccine materials versus highly virulent or antibiotic resistance pathogens). This signature level involves some 20 to 30 primer pairs. Together, the primer pairs offer a certainty of correct identification. Currently, providing such a high level of confidence requires several days; the goal again is to reduce the time to less than 30 minutes.

The final signature level, intended primarily for law enforcement use, will permit detailed identification of a specific strain of a pathogen (for example, *Yersinia pestis* KIM) and correlate that strain with other forensic evidence. Such data will help to identify and prosecute attackers. The present typical time lag for results is currently a few weeks, and the goal is to reduce that to a few days.

Biological foundations program scientists have worked with DOE and other agencies to assemble a list of natural pathogens most likely to be used in a

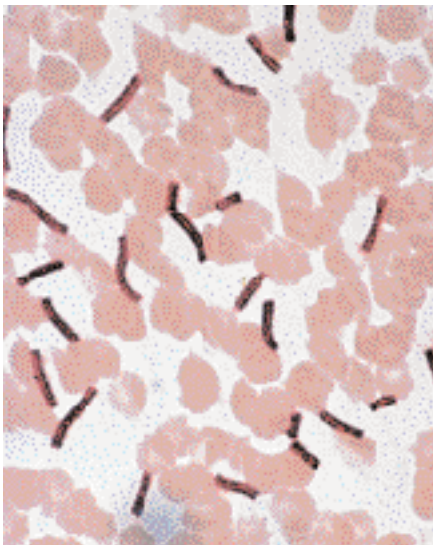


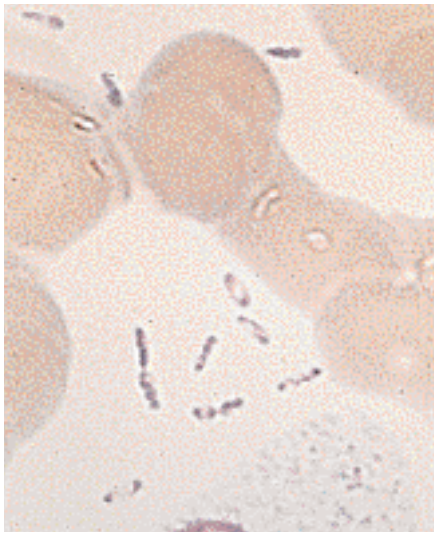
Bacterial chromosomes (DNA) form loops, unlike human chromosomes which form strands. In the loop, between two to five million bases of bacterial DNA are screened to locate unique regions (circled), which are marked with primer pairs. The marked regions are amplified thousands of times using polymerase chain reaction technology and then processed to identify and characterize an organism.



Different levels of signature complexity provide different problem resolutions. A simple signature, involving only one or two unique DNA marker regions, provides genus-level identification. Signatures for species- and strain-level identifications involve more marker regions and take longer to process, but provide more detail and accuracy. Law enforcement uses require signatures that provide strain-level identification.

domestic attack. The list includes bacteria, viruses, and other classes of threats, such as agricultural pathogens. Two extremely virulent pathogens head the list: *B. anthracis* and *Y. pestis*, which cause anthrax and plague in humans, respectively. *Bacillus anthracis* has few detectable differences among its strains, whereas *Y. pestis* strains can vary considerably in genetic makeup. Unraveling the significant differences between the two organisms will give national laboratory researchers experience vital for facing the challenges of the next few years, as they develop signatures for a wide spectrum of microbes.





Two extremely virulent organisms head the list of pathogens most likely to be used by terrorists: *B. anthracis* (top) and *Y. pestis* (bottom), which cause anthrax and plague in humans, respectively.

Livermore Focuses on Plague

Research has been divided and is carefully coordinated among laboratories to avoid duplication. Livermore researchers are focusing on *Y. pestis*, *Francisella tularensis* (a bacterium causing a plaguelike illness in humans), and several other microbes that threaten human and animal health. They are working in collaboration with the U.S. Army Medical Research Institute of Infectious Diseases, the Centers for Disease Control and Prevention, the California Department of Health Services, Louisiana State University, Michigan State University, and research centers in France, China, and Russia. "We want to be prepared for the most likely pathogens from throughout the world," says Weinstein.

Eleven species and many thousands of strains belong to the *Yersinia* genus. The most notorious species, *Y. pestis*, causes bubonic plague and is usually fatal unless treated quickly with antibiotics. The disease is transmitted by rodents and their fleas to humans and other animals. Although rare in the U.S., cases are still reported in the Southwest.

Livermore researcher Emilio Garcia notes that the seemingly subtle DNA differences among many *Yersinia* species mask important differences. One species causes gastroenteritis, another is often fatal, and a third is virtually harmless; yet all have very similar genetic makeup. Garcia's team is using a technique called insertion-sequence-based fingerprinting to understand these slight genetic differences. Insertion sequences are mobile sections of DNA that replicate on their own. Analyzing for their presence will

Biological Warfare Has a Long History

The use of biological agents as weapons is not a new phenomenon, Lawrence Livermore's Tony Carrano points out. The Romans, for example, used corpses of diseased animals to poison the drinking wells of their enemies. During the horrific Black Death of the Middle Ages, the bodies of bubonic plague victims were catapulted over fortress walls of besieged cities.

During the French and Indian wars, 1754–1763, the British gave smallpox-infested blankets as gifts to the Indians because of their suspected alliance with the French. During World War II, Germany and Japan produced bacteria capable of infecting humans.

Biological attacks in the United States have been few and isolated. One occurred in 1984, when followers of Baghwan Shree Rajneesh poisoned several salad bars in Oregon with salmonella bacteria. In Europe, terrorist groups in Germany began producing botulinum toxin. In the late 1980s in Japan, the Aum Shinrikyo cult acquired anthrax bacteria and botulinum toxin and attempted to collect samples of Ebola virus.

Following the 1991 Persian Gulf War, United Nations inspectors revealed the vast scope of Iraq's biological arsenal. Iraq was found to possess more than 150 bombs and 25 missile warheads filled with botulinum toxin, anthrax, or aflatoxin. What's more, Iraq had built sophisticated laboratories to study and produce a wide range of biological agents and toxins.

not only help refine signatures for *Y. pestis* but also shed light on how microorganisms evolve into strains that produce lethal toxins. This understanding, in turn, should give ammunition to researchers seeking an antidote or vaccine.

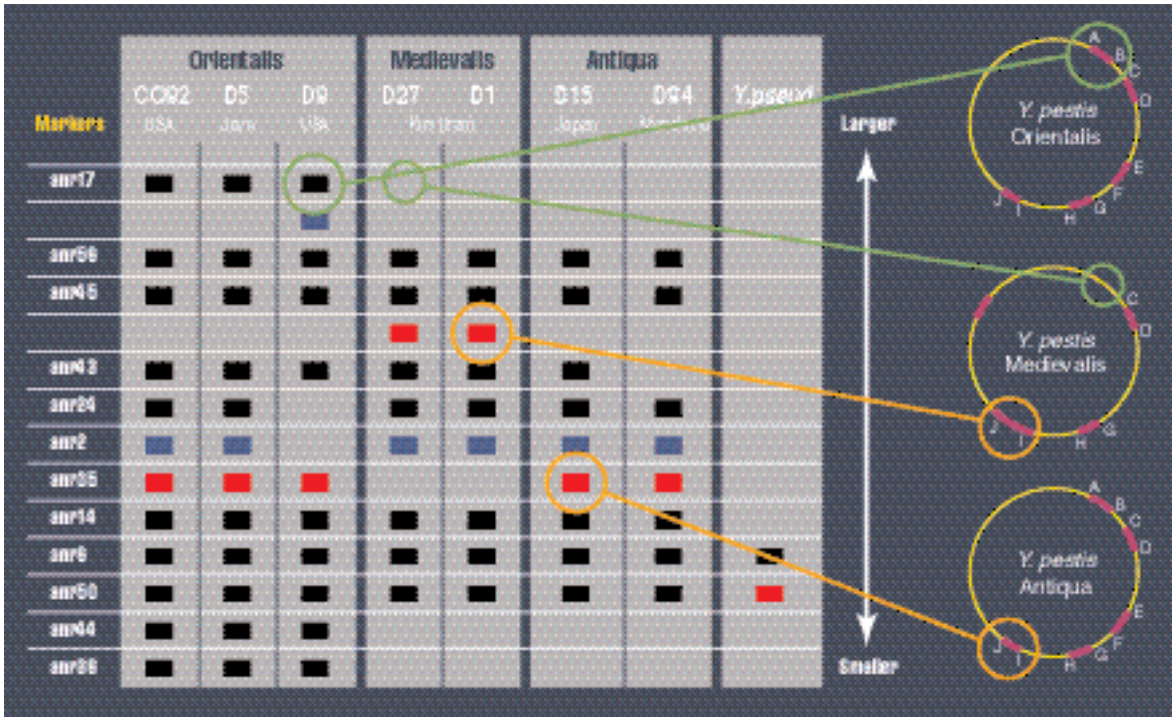
Garcia's team is collaborating with other world-renowned research centers to better understand the genetic differences among species and strains. A collaboration with France's Pasteur Institute is comparing the genetic complement of *Y. pestis* with another member of the *Yersinia* group (*pseudotuberculosis*) that causes an intestinal disease. "They are closely related, and yet they cause such different diseases," Garcia says.

Better and Faster, with More Uses

Livermore scientists are using a number of methods that allow more rapid identification and characterization of unique segments of DNA. Each method has advantages and drawbacks, with some more applicable to one organism than another. Weinstein expects that within two years, the Livermore team will have settled on a handful of techniques as the workhorses of signature generation.

In addition to the insertion sequence method, another promising technique is called suppressive subtractive hybridization. The method takes an organism and its near neighbor, hybridizes the DNA from both, and determines the fragments not in common as the basis of a signature. A team headed by Lawrence Livermore biomedical scientist Gary Andersen is working with colleagues at Moscow State University in Russia to advance the technique; one goal is to simultaneously analyze 96 strains of DNA.

Andersen's team has used suppressive subtractive hybridization to distinguish the DNA of *Y. pestis* from that of *Y. pseudotuberculosis*. The team has also used the technique to aid California's poultry industry by providing a handy way to detect *Salmonella enteritidis*. This bacterium can cause illness if eggs are eaten raw or undercooked. Subtractive hybridization results have been so successful that the



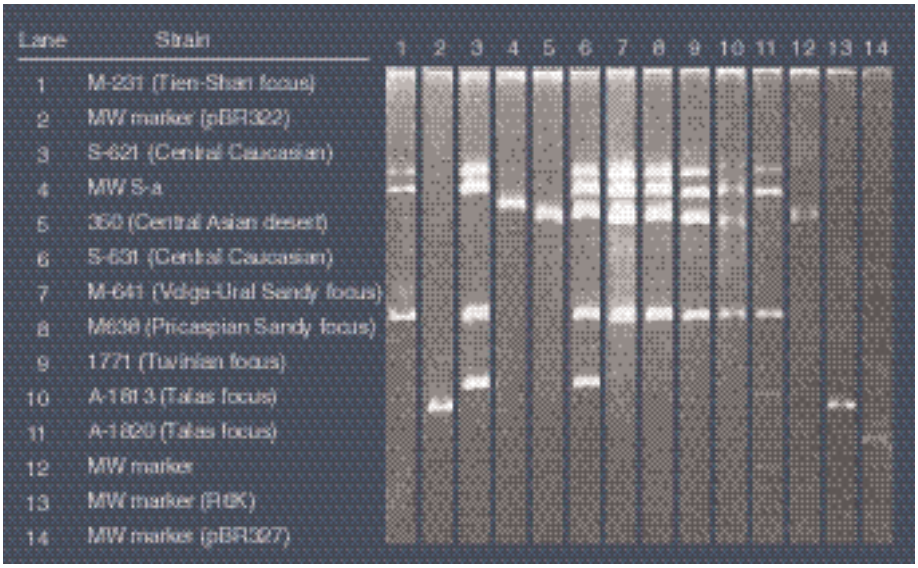
Insertion sequences are repeated sections of DNA whose location in the chromosome varies between different strains. Analyzing for their presence provides information about the type and biological function of a strain. The table at above left shows differences in the insertion sequence "fingerprints" of *Y. pestis* strains associated with the last three plague outbreaks. Red and blue rectangles indicate fragment shifts and changes from strain to strain. Some of these differences are graphically represented in the three strains of *Y. pestis* diagrammed at above right. For example, a fragment found in Orientalis is absent from Medievalis, and a fragment in Antiqua has shifted and become shorter than what it was in Medievalis.

signature can now be used to distinguish between subtypes of salmonella bacterium.

In addition to the DNA-based pathogen detection methods, researchers are developing detection capabilities using antibodies that can tag a pathogen by attaching to a molecular-level physical feature of the organism. Antibody assays are likely to play an important role in pathogen detection because they are generally fast and easy to use (commercial home-use medical tests use this form of assay).

Biological foundation researchers are working to improve these detection methods as well. For example, a collaboration with the Saratov Anti-Plague Institute in Russia is studying a bacteriophage (bacteria-killing virus) that only attacks *Y. pestis* and none of its cousins. Researchers recently discovered that the virus produces a unique protein component to attach to the bacterium cell wall at a certain site and gain entry. Garcia says that recognizing the distinct site could form the basis of a foolproof antibody signature. "If it's possible to achieve it with *Y. pestis*, we may be able to do it with other pathogens," he adds.

A Lawrence Livermore team has aided California's poultry industry with a biological signature to detect *Salmonella enteritidis*, a bacterium that can cause illness if eggs are eaten raw or undercooked. The signature can distinguish between subtypes of the bacterium and their different pathways to humans and other hosts.



Russia's Saratov Anti-Plague Institute is an important collaborator with Livermore in elucidating the subtle genetic differences among strains of *Y. pestis*. Above are some of the strains isolated by the institute.

Sensing Virulence

As more information about pathogens and their disease mechanisms becomes available and as genetic engineering tools to transplant genes become cheaper and simpler to use, the threat of genetically engineered pathogens increases. Biodetectors must be able to sense the virulence signatures of genetically engineered pathogens, or they will be blind to an entire class of threats. "Our ultimate objective is to identify several specific virulence factors that might be used in engineered biological warfare organisms so that we can detect these engineered organisms and break their virulence pathway," says Weinstein.

One key factor useful for detecting engineered organisms is an antibiotic resistance gene. When transplanted into an infectious microbe, the gene could greatly increase the effectiveness of a biological attack and complicate medical response. Some antibiotic resistance genes are widely shared among bacteria and are easily transferred with elementary molecular biology methods. In fact, a standard biotechnology research technique is introducing antibiotic resistance genes into bacteria as an indicator of successful cloning. "We need to be able to rapidly recognize such genes so that the medical response is appropriate," says Weinstein.

Another telltale indication of genetic tampering is the presence of virulence genes in a microbe that should not contain them. Virulence genes are often involved in producing toxins or molecules that cause harm or that simply evade a host's defense. "If a series of genes is made available to perform their functions at the right time, they could cause real damage," says Lawrence Livermore molecular geneticist Paula McCready. If interfering with the action of one of these genes or its proteins interrupts the virulence pathway, the disease process can be halted. Identifying and characterizing important virulence genes and determining their detailed molecular structure will greatly aid the development of vaccines, drugs, and other medical treatments.

As an example, *Y. pestis* disables the immune system in humans by injecting proteins into macrophages, one of the body's key defenders against bacterial attack. Because the protein acts as an immunosuppressant to disable the macrophage, understanding its structure not only would help scientists fashion a drug that physically blocks the protein but also would shed light on autoimmune diseases such as arthritis and asthma. A Lawrence Livermore team led by Rod Balhorn is working to determine the three-dimensional shapes of toxins such as the one produced by *Y. pestis* (see *S&TR*, April 1999, pp. 4–9).

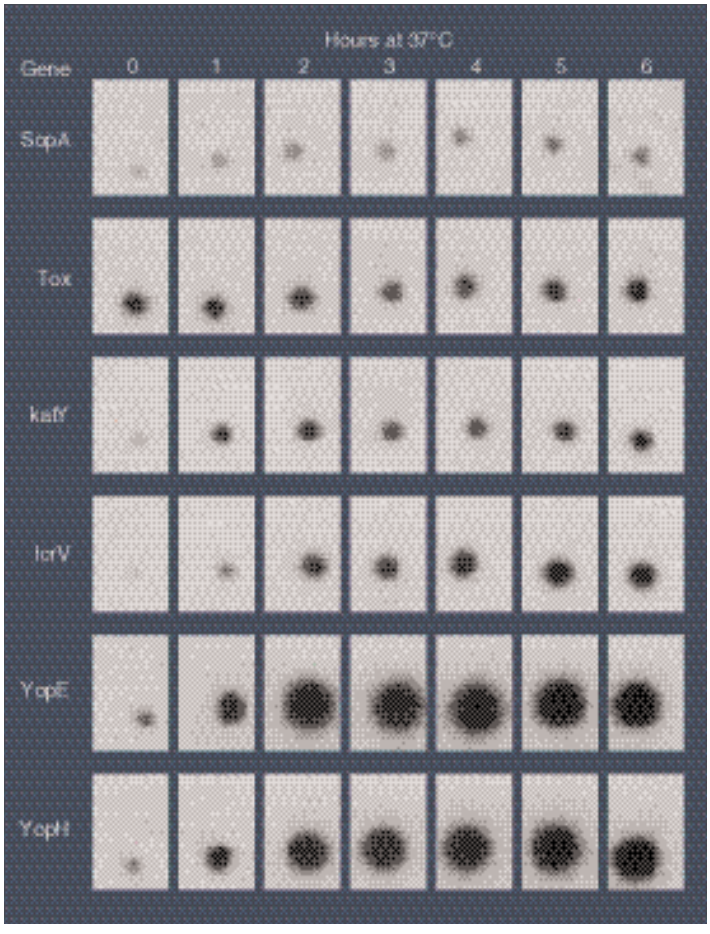
Virulence Genes in Common

Virulence genes spread naturally among pathogens and thus are also found in unrelated microbial species. Therefore, virulence genes alone are not sufficient for species-specific DNA-based detection. "We have to differentiate the virulence genes in natural organisms from engineered organisms," says Garcia.

Livermore researchers are using different methods for differentiating virulence genes from among the thousands of genes comprising the genomes of pathogens. One technique looks for genes that "switch on" (start making proteins) at the internal temperatures of mammals. For example, Livermore scientists are studying genes of *Y. pestis* that become much more active at 37°C. It seems a safe bet that many of these genes are associated with the bacterium multiplying within a warm-blooded host.

In 1998, a Lawrence Livermore team made an important contribution to understanding the genetics of *Y. pestis.* They sequenced the three plasmids (bits of DNA located outside the microorganism's circular chromosome) that contain most of the virulence genes required for full development of the bubonic plague in animals and humans. Plasmids sometimes transfer their genes to neighboring bacteria in what is called lateral evolution. (Antibiotic resistance genes are also located on plasmids.)

Garcia, who led the plasmid sequencing team, says that studying virulence genes can shed light on how new strains develop. The *Y. pestis* strain that causes bubonic plague, for example, may have evolved some 20,000 years ago. Such understanding is relevant to HIV, which may not have become infectious for humans until the 20th century.



As a way of identifying virulence genes, Livermore researchers look for bacterial genes that produce proteins at the internal temperatures of mammals (37°C). Four such genes (katY, lcrV, YopE, and YopH) in *Y. pestis* become much more active over a 6-hour period at 37°C.

Working with End Users

McCready notes that there needs to be a strong relationship between development of biological signatures and detection technologies and their end uses. Livermore researchers work with agencies that will be using signatures from Livermore and Los Alamos for both handheld detectors and field laboratories. "We want to make sure our tools get to the experts and agencies that need them," she says.

McCready is working closely with colleagues at the Bioterrorism Rapid Response and Advanced Technology Laboratory of the federal Centers for Disease Control and Prevention. Livermore is collaborating with the CDC to make diagnostic tools available to regional public health agencies and thus create a national mechanism for responding quickly to bioterrorism threats. Currently, many health agencies use detection methods that are not sufficiently sensitive, selective, or fast. For example, one culture test for detecting anthrax takes two days. Major damage and even death may have occurred in that time.

McCready emphasizes that DNA signatures will be thoroughly validated before being released, because their use might lead to evacuations of subways, airports, or sporting events, and such evacuations cannot be undertaken lightly. As part of the validation effort, Livermore scientists are characterizing natural microbial backgrounds to make sure that the signatures are accurate under actual conditions. To that end, researchers are collecting background microbial samples in air, water, and soil, as well as in human blood, urine, and saliva. McCready points out that *B. anthracis* is related to *B. thruginensis*, a naturally occurring harmless microbe that lives in dirt and can give a false positive reading to anthrax if the signature used is not adequately specific. The characterization effort is being aided by a device called the Gene Chip. Manufactured by Affymetrix Inc. and using technology developed by Livermore, the device simultaneously monitors the expression of thousands of genes.

Livermore researchers are looking ahead to a time when their efforts will have helped to equip federal and state agencies with a robust set of biological

signatures crucial for America's response to any biological warfare threat. Equally important, the researchers envision a strong mechanism linking biomedical scientists with public health and law enforcement officials to develop new signatures speedily and cost-effectively to stay several steps ahead of terrorists.

—Arnie Heller

Key Words: anthrax, bacteriophage, biological signatures, biological weapons, Centers for Disease Control and Prevention (CDC), DNA, Gene Chip, plague, plasmids, virulence.

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About the Scientist

BERT WEINSTEIN is the deputy associate director of Livermore's Biology and Biotechnology Research Program (BBRP) Directorate. He received his B.S. in physics and mathematics from Brigham Young University and his M.S. and Ph.D. in physics from the University of Illinois at Urbana. He currently serves as leader of the biological foundations thrust area for the DOE Chemical and Biological Nonproliferation Program and as liaison for BBRP with the DOE Joint Genome Institute in Walnut Creek, California. At the Laboratory since 1974, he has held both research and leadership positions in four major programs: inertial confinement fusion, nuclear design, intelligence and national security, and the biology and biotechnology research program. He also served as a member of the Science Council for the Department of Energy's Office of Nonproliferation and National Security (now the Office of Defense Nuclear Nonproliferation).

Biomedical Research Benefits

It's no fluke. An experiment that can track 35 micrograms of folic acid through a human for 200 days is routine for Livermore's Center for Accelerator Mass Spectrometry.

Members of Livermore's core biological AMS team stand in front of the new, small spectrometer (also seen in the larger photo) dedicated to biological studies using carbon-14. From left: John Knezovich, Bruce Buchholz, Graham Bench, Ken Turteltaub, John Vogel, Ted Ognibene, and Mark Roberts. Missing from the group is Karen Dingley.

THE short history of accelerator mass spectrometry (AMS) for biomedical research at Livermore has been sweet indeed. Just 10 years ago, Livermore scientists first used AMS to determine how low doses of a suspected carcinogen affect the DNA of mice. A remarkably sensitive measuring technique, AMS can seek out one carbon-14 isotope from among a quadrillion other carbon atoms. It achieved a tenfold improvement in detecting damaged DNA over the best methods then available, thus enabling studies to be conducted directly with humans. Ever since, Livermore has been expanding the development of AMS for biomedical and pharmaceutical applications and is today a recognized leader in the field. (See box on p. 14 for information on how AMS works.)

Livermore researchers are continuing to study the effects of carcinogens on humans and animals. Perhaps not surprisingly, they have discovered that humans and animals metabolize these substances differently, with resulting differences in the way DNA is affected. AMS is being used in collaborations with researchers from around the world to begin to solve many challenges in biomedicine—from examining the way we metabolize vitamins to developing a new cancer diagnostic test. AMS has even proved invaluable for learning how pesticides move through ant colonies from workers to the queen.

When Livermore proposed that the National Institutes of Health (NIH) establish a National Research Resource for AMS at Livermore, University of California (UC) and Department of Energy scientists provided key testimony to demonstrate the value of AMS. Along with all the other evidence, their testimony must have been persuasive. Last November, Lawrence Livermore joined an elite group of research facilities when NIH

from Counting Small

awarded the five-year grant. There are other NIH Research Resource facilities for various types of mass spectrometry, but this is the first for AMS. An NIH review of Livermore's proposal had this to say:

> *An overwhelming case was made for the need for an AMS resource and a number of outstanding collaborative projects have already been initiated. . . . At the present time, the LLNL Resource is clearly the most advanced site in the U.S. to explore the use of AMS in biomedical research.*

Chemist John Knezovich, director of Livermore's Center for Accelerator Mass Spectrometry (CAMS), is pleased with progress to date. "CAMS is unique in this country in concentrating on biological AMS. Beyond integrating the Laboratory's scientific expertise in both AMS and biomedical research, CAMS provides a major facility that is enabling research projects from all over the world."

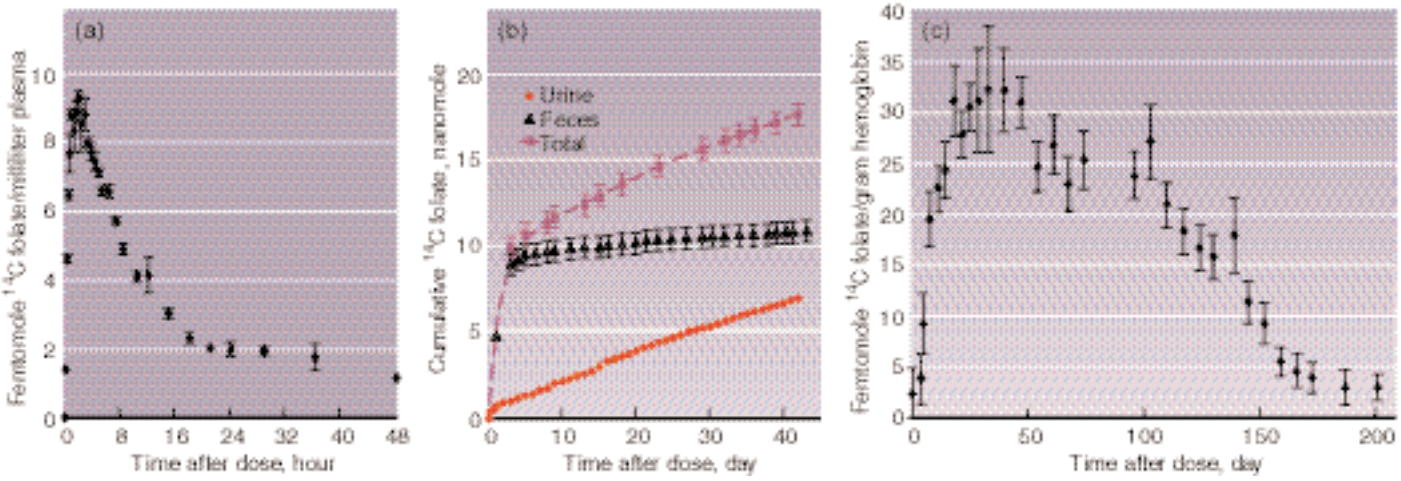
He adds, "A big plus is that in addition to the large, multipurpose AMS machine that we've had for years, a much smaller one will soon come on line that is dedicated to biological studies using carbon-14. And, we have begun to use yet another new spectrometer for biological samples that have been tagged with tritium, a new tracer element for AMS. On top of that, we have added a heavy-isotope line to our large AMS machine for studies of plutonium." (See box on p. 17 for more information on AMS equipment at Livermore.)

An important aspect of Livermore's work to date has been in establishing AMS as a routine biomedical research tool. An AMS experiment no longer requires a large and expensive staff of physicists and technicians. Livermore scientists have led the technological advances necessary to make AMS a more effective, dependable, and economic tool for the biomedical, pharmaceutical, chemical, and clinical communities (see also *S&TR*, November 1997, pp. 4–11).

Established in 1989, CAMS was designed to diagnose the fission products of atomic tests; monitor the spread of nuclear weapons to other countries by detecting telltale radioisotopes in air, water, and soil samples; and use isotopic tracers to study climate and geologic records. It still does all these things today—and much, much more.

Sensitivity Is the Key

So what is the value of AMS to you and me? AMS is an ideal method for tracing the passage of chemicals through humans without disturbing normal metabolic processes. Perhaps researchers want to know how the human body metabolizes a drug or vitamin. The molecules of the substance are manipulated slightly to "tag" them with a radioactive isotope, typically carbon-14, though other radioisotopes may be used as well. Rare radioisotopes of elements found in organic materials are used as tracers because they can be incorporated



A single dose of carbon-14-tagged folic acid was traced for 200 days. (a) The tagged folic acid appears very quickly in plasma (the liquid part of blood) and tapers off in about two days. (b) The amounts of tagged folate being eliminated in feces and urine were followed for 40 days. (c) Folate begins to be incorporated into hemoglobin at day 5. (Hemoglobin is the iron-containing, oxygen-carrying molecule in red blood cells.) The level of folate in hemoglobin peaks at about the 30th day and disappears only after 200 days.

into biomolecules and because they are present naturally at low levels, so tagged molecules can be detected easily.

A collection of human subjects swallows or otherwise ingests the substance. Then, using AMS to measure the number of carbon-14 atoms in samples of urine, feces, saliva, or blood over the next hours, days, and weeks, researchers can trace how much of the substance is absorbed, how it travels through the body, what organs it affects, how much of it is lost through excretion, and so on. The first experiment to trace the vitamin folic acid in a human followed a single dose of just 35 micrograms for a remarkable 200 days.

Information this detailed has never before been available using healthy human subjects. Less sensitive measuring techniques, such as scintillation counting, require ingesting large doses of both the

Accelerator Mass Spectrometry at Livermore

Livermore's Center for Accelerator Mass Spectrometry (CAMS) is home to the most versatile and productive AMS facility in the world. AMS is an exceptionally sensitive technique for measuring concentrations of isotopes in small samples, typically less than 1 milligram, and the relative abundance of isotopes at low levels. It can, for example, find one carbon-14 isotope among a quadrillion other carbon atoms.

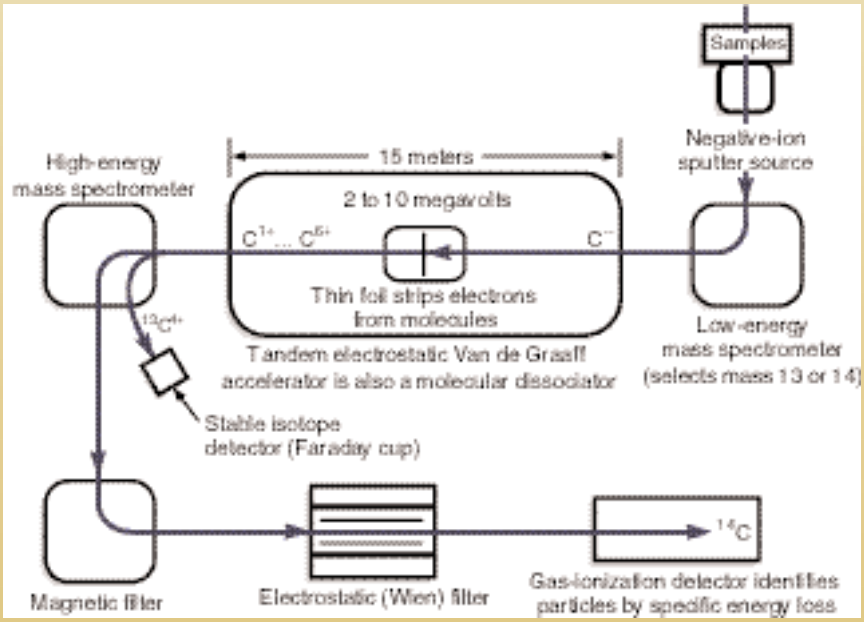
Mass spectrometry has been used since early in the 1900s to study the chemical makeup of substances. A sample is put into a mass spectrometer, which ionizes it and analyzes the motion of the various ions in an electric field to sort them out by their mass-to-charge ratios. The basic principle is that isotopes of different masses move differently in a given electromagnetic field.

In accelerator mass spectrometry, the same principle applies but the process is different. Negative ions made in an ion source are accelerated in a field of millions of volts. The accelerated ions smash through a thin carbon foil or gas that destroys all molecular species. After passing through a high-energy mass spectrometer and various filters, the ions finally slow to a stop in a gas ionization detector. (See the layout at right of the large AMS machine at Livermore.) The identity of individual ions can be determined from how the ions slow down. For example, carbon-14 slows down more slowly than nitrogen-14, so those ions of the same mass can be distinguished from one another. Once the charges are determined, the detector can tell to which element each ion belongs and counts the desired isotope as a ratio of a more abundant isotope—carbon-14 as a ratio of carbon-13, for example.

The two "tricks" that make AMS work so well are the molecular dissociation process that occurs in the accelerator and the ion detection at the end. The resulting sensitivity is typically a million times greater than that of conventional isotopic detection.

For biological studies, AMS has been used primarily for counting carbon-14 because carbon is present in most molecules of biological interest and carbon-14 is relatively rare in the biosphere. Tritium (hydrogen-3) has also been used extensively as a tracer in biological research. The use of tritium in AMS is new and holds great promise, because many than with carbon-14. Other isotopes are measured by AMS as well, including plutonium-239, calcium-41, beryllium-10, chlorine-36, and iodine-129.

All over the world, AMS is still used primarily to count carbon-14 in archaeological and geologic samples for dating purposes. In the 1980s, it replaced the traditional method of scintillation counting for precise radiocarbon dating, which was time-consuming and required relatively large samples. Livermore performs radiocarbon dating and many other forms of AMS 24 hours a day, 7 days a week for its own research and collaborations as well as for others on a fee-for-service basis.



chemical being studied and the radioisotope, something few people want to do. Sometimes, such studies are done with volunteers. Usually, however, scientists have used animals for their research. Then considerable extrapolating has been required: from large doses to small doses and from laboratory animals to humans.

Now researchers can use much smaller, more realistic doses on human subjects. They can measure the true effects of a typical dose of, say, vitamin A or aspirin. AMS, the only method that can trace these low doses over such long time periods, has been described as the most significant new tool for nutritional studies since the 1930s.

And the amount of radioactivity taken in with the chemical being studied is less than one would encounter during a single day's exposure to background ionizing radiation from walking around in the sunshine. (Cosmic rays contain a small amount of radioactivity that we are exposed to every day.) An airplane flight exposes us to far more ionizing radiation than participation in one of these experiments.

Biochemist Ken Turteltaub, one of the developers of AMS for biological applications, is sold on the process. He says, "With accelerator mass spectrometry, we can address problems that cannot be solved otherwise."

Grant Recognizes Achievement

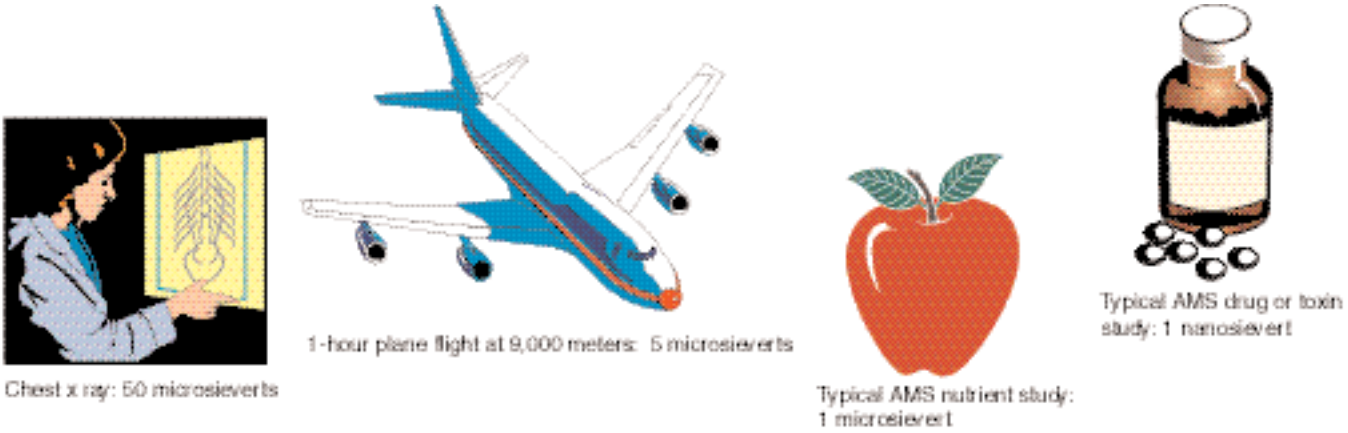
The Livermore team spent five years building and demonstrating the capabilities that led to the NIH grant to Livermore. Today, Turteltaub is the grant's principal investigator, assisted by fellow biochemist Karen Dingley and a team of CAMS scientists. As an NIH Research Resource for biological AMS, Livermore is charged with providing biological researchers throughout the country with access to carbon-14 AMS analysis in their research, developing new methods and instrumentation for the use of AMS in biomedical research, demonstrating new applications, and educating the biomedical research community on AMS. All of these functions have been under way at Livermore for many years, which makes award of the grant particularly gratifying as recognition of a job well done.

All NIH Research Resource grants focus on collaborative work in order to educate the biosciences community. For this grant, Turteltaub and his cohorts are working to expand support for the use of AMS and to export its use to as many researchers as possible.

In work that is just beginning, they are also developing new experimental methods so that AMS can be applied to as many types of biological research as possible. They are developing the sample preparation and analytical methods necessary to reduce handling, automate processes, and increase sample throughput. The team will explore new spectrometer components that allow the direct interface of bioanalytical instrumentation to the spectrometer for simplified, rapid, on-line analysis. Finally, further development of the small carbon-14 spectrometer will help to bring AMS machines to more bioanalytical laboratories.

Measuring Damage to Molecules

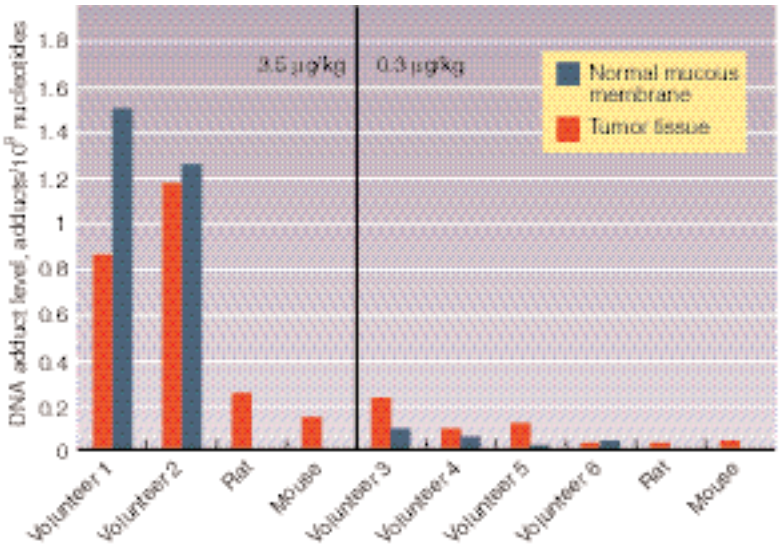
Turteltaub, Dingley, and other researchers at Livermore are already involved in several collaborations with researchers in the U.S. and England to examine the effects of substances produced by cooking meat. Both 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) are heterocyclic aromatic amines that have been shown to cause cancer in laboratory animals when



Taking a 1-hour plane trip exposes a person to 5,000 times more ionizing radiation than participating in an accelerator mass spectrometry study of a drug or toxin. Everyone on Earth takes in about 2.5 millisieverts of radiation every year. (A sievert is a Système International [SI] unit for radiation dose. It is equal to 8.38 roentgens. One microsievert is one millionth of a sievert and a nanosievert is one billionth of a sievert.)

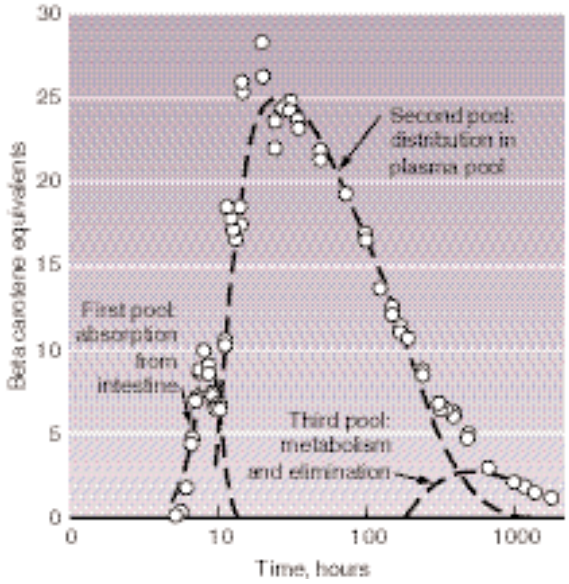
administered at high doses. The team has used AMS to establish whether DNA and protein adducts (damage) can be detected in laboratory animals and humans when they take in a smaller, more typical dietary amount of these substances.

In numerous experiments using carbon-14-tagged PhIP and MeIQx molecules, the team has confirmed not only that adducts can be detected at low doses, but also that humans may be more sensitive to these substances than mice or rats. These results are important because researchers in the fields of toxicology, pharmacology, and nutrition currently make two basic assumptions: that data obtained from high-dose experiments can be accurately extrapolated to more typical environmental levels and that animal models are valid. While all the data are not yet in on the veracity of either assumption, AMS provides the sensitivity and precision needed to address each of them thoroughly.



Rodents and humans exhibit significantly different responses to high and low doses of MeIQx, a chemical that appears in meat after it is cooked. MeIQx causes damage to DNA known as adducts.

Three "pools" of carbon-14-tagged beta carotene can be identified from blood, urine, and feces collected for 3 months after ingestion. The first peak is due to carotene metabolizing to retinol in the intestine, from where it is quickly excreted. The largest pool in plasma reaches its highest point a day after dosing but is then drawn into a deeper pool, perhaps the muscular tissue, over the next 5 to 10 days. This pool is slowly metabolized back to a circulating form and finally, in the third pool, is eliminated.



Dingley performed the first AMS experiments using biomolecules labeled with tritium (hydrogen-3). With tritium-labeled PhIP and carbon-14-labeled MeIQx, she found comparable sensitivities at low doses. (The lowest doses were equivalent to the amount of PhIP you would take in when eating a single well-cooked hamburger.) These experiments demonstrated the feasibility of using tritium AMS for biomedical studies as well as the feasibility of using both tracers in experiments to study how two substances, given at once, may affect each other.

Tritium is widely used in biological tracing and has some advantages over carbon-14. It is relatively easy to label a molecule with tritium, whereas carbon-14-labeled molecules must be custom synthesized, which can be expensive. While the use of detection techniques other than AMS requires relatively high tritium dosages and large samples, AMS eliminates those disadvantages. In fact, Livermore's experiments have demonstrated that AMS could be used to detect tritium in biological samples with a 100- to 1,000-fold improvement over scintillation and other decay counting techniques.

From Ants to Elephants

AMS is ideal for measuring extremely small samples, certainly the case when ants are the subject of study. CAMS worked with UC Riverside to follow the path of carbon-14-tagged food and insecticides through colonies

Research Opportunities Grow with New and Better Equipment

A continuing thrust of the Center for Accelerator Mass Spectrometry (CAMS) at Livermore has been the development and application of new AMS technologies as well as the improvement of existing ones. Three entirely new systems for measuring carbon-14, tritium (hydrogen-3), and plutonium-239 have come on line in recent weeks and months. Tweaking of the 15-meter-long, multipurpose AMS unit, in place since the inception of CAMS in 1989, never stops. After 11 years of development, the unit's cesium ion source is the most powerful in the world. Sample preparation methods and experimental protocols evolve and are standardized as Livermore's capabilities grow and as the demands of the biological research community expand. The focus now is to robotize the sample preparation process as much as possible.

For several years, researchers at Livermore have been working to develop a smaller, less expensive spectrometer dedicated to carbon-14 biological research. Biological AMS experiments can be run more quickly than most other AMS applications such as radiocarbon dating. A dedicated bioresearch unit would free the larger machine for other AMS work.

Testing for the first such small unit began in June (see photo on p. 12). One-tenth the size of the larger machine, the small spectrometer will provide higher throughput for biological samples. It also will serve as a testbed for new sample preparation and delivery technologies.

John Knezovich, director of CAMS, says, "These smaller units are just beginning to appear. Livermore is one of four institutions in the world using one. Now, virtually every AMS unit, of whatever size, is custom-built. But when they have been developed to the point where the cost for one is below $1 million, then we can expect to see a proliferation of small AMS machines operating in universities and pharmaceutical companies, making significant scientific advances."

Livermore's new tritium system (below left) is the result of a collaboration with AccSys Technology of Pleasanton, California, and was funded by a National Institutes of Health/National Cancer Institute Small Business Innovation Research grant. The system was specifically designed to be small, simple, and inexpensive, making the technique of tritium AMS more broadly accessible.

Physicist Mark Roberts was primarily responsible for the system's design and for bringing it on line. Biochemist Karen Dingley, who performed the first AMS experiments using tritium as a biological tracer, has been developing experimental methods, including sample preparation and handling protocols. One of the system's first uses is in experiments to improve current methods to measure the rate at which cells divide. Cancer is a disease in which cells divide uncontrollably. Researchers want to know if exposure to cancer-causing chemicals results in an increased rate of cell division before a tumor appears. Normally, experiments such as this would require large doses of toxic chemicals or radioactive isotopes. With AMS, only a very small amount of tritium in drinking water does the trick. Then, the tritium in newly synthesized DNA can be measured. The level of tritium incorporation in DNA indicates the approximate rate at which new cells are synthesized and thus the rate of cell division.

Physicist Jeff McAnich has led the development of a new heavy-isotope spectrometer (below right) that makes it easier to run plutonium samples on a routine basis. The initial focus of this work is the measurement of plutonium concentrations and isotope ratios in urine bioassays of Marshall Islanders who are being resettled to Bikini Atoll and other islands used in the 1950s for weapons testing. The unit will be able to measure plutonium-239/240 as well as plutonium-241. Other heavy isotopes such as neptunium-237 may also be studied in the future.

Livermore's Center for Accelerator Mass Spectrometry has developed specialized spectrometers for tritium (photo at left), heavy isotopes such as plutonium (photo at right), and carbon-14 (see p. 12), in addition to its large, multipurpose machine.

of Argentine ants housed at the university. Once native to Brazil and Argentina, the Argentine ant is now the most prevalent pest around homes in California, the Caribbean, the Mediterranean, and South Africa. No available control strategy works effectively because each colony is home to several queens, any of which can regenerate the colony if other queens are destroyed. Learning how nutrients make their way to the queens is essential to finding an effective control method.

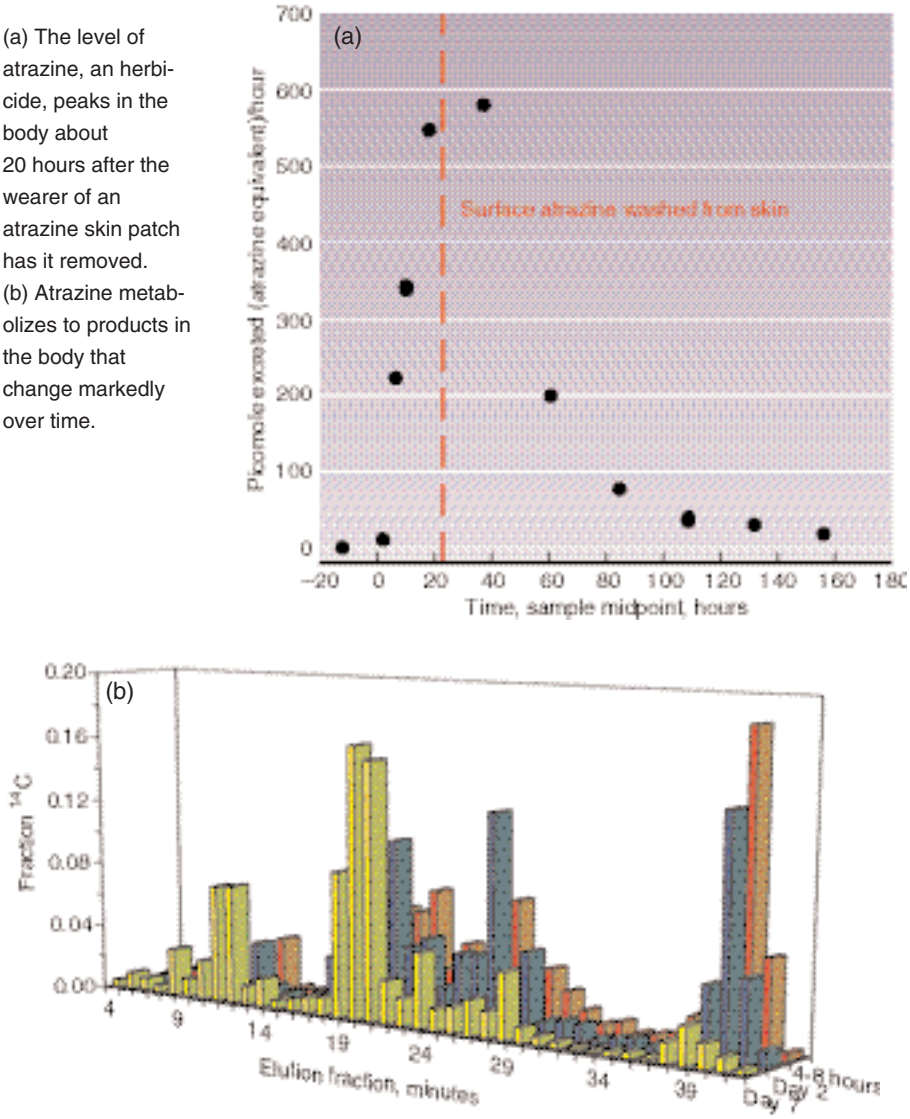
Work on nutrient dynamics was part of the largest AMS collaboration in place before the new NIH Research Resource was established. It involves CAMS and four UC campuses. Several campus—lab collaborations are ongoing at Livermore and other laboratories managed by UC, and the UC Office of the President has described this one as the most successful of all. Livermore's principal investigator for the Consortium for Ultra-Low-Level Tracing is physicist John Vogel, assisted by Bruce Buchholz, who was trained as a nuclear engineer.

Vogel has been involved with CAMS from the start and has been responsible for many of the technological advances that have helped to establish AMS as an increasingly routine biomedical research tool. His work with UC Davis on nutritional studies has earned him the rank of adjunct full professor of nutrition. "One never knows where a career will go," he chuckles.

The CAMS–UC Davis experiment that followed a single dose of folic acid for 200 days was no fluke. Many over-the-counter products that we take with barely a thought stay in our bodies for a remarkably long time. A similar experiment followed a single dose of beta carotene in blood, urine, and feces samples for 3 months. The three primary "pools" of beta carotene or retinol, the vitamin A that beta carotene metabolizes to, are clearly visible, first in the intestine, then in blood plasma, and finally in a circulating form with a 38-day half-life. The existence and equivalence of the slowly changing pool that starts 9 days after dosing would be undetectable without AMS.

Metabolic studies of vitamins and other nutrients are of more than passing academic interest. At present, little is known about vitamin metabolism except that it seems to vary widely among individuals. Folic acid, one of the B vitamins, is required for the production of red blood cells, DNA, and RNA. A deficiency can cause anemia and is related to heart disease and certain birth defects. A deficiency in beta carotene can cause blindness, a serious problem in less developed parts of the world. In fact, a new variety of carroty-orange "golden" rice, rich in beta carotene, has recently been developed in Switzerland for use in undeveloped countries.

Dr. Andy Clifford, professor of nutrition at UC Davis, has headed all of this important nutrient metabo-



(a) The level of atrazine, an herbicide, peaks in the body about 20 hours after the wearer of an atrazine skin patch has it removed. (b) Atrazine metabolizes to products in the body that change markedly over time.

In 1998, a University of California at Riverside doctoral candidate won the annual award given by the *International Journal of Mass Spectrometry and Ion Processes* for best student paper. Her fundamental work in protein chemistry netted her a trip to Amsterdam as well as a cash prize. She could not have won without Livermore's biological AMS facilities because her subject, nematodes, supplied only the smallest of samples.

lism work with CAMS. As a result of his research to date, he has been awarded follow-on grants from NIH to conduct definitive AMS studies in humans. His experiments are now under way using healthy human volunteers aged 18 to 60.

CAMS researchers are working with clinicians at UC San Diego to develop a diagnostic technique sensitive enough to detect the growth protein that cancerous tumors produce. Most of this protein stays at the site of the tumor, but a very small amount leaks out into the blood. Different kinds of tumors—ovarian, prostate, breast, liver, and so on—produce slightly different kinds of growth proteins. A blood test that could detect the protein would be an effective tool for an early diagnosis. The test could also be used to determine whether follow-up treatment was working; ideally, the protein level would drop to zero over time.

Yet another current study is of very large animals—elephants—but small samples. A hormone imbalance in bull elephants can produce raging behavior known as musth. The only way to obtain a sample from an enraged elephant is from its feces. But most of that is undigested fiber, leaving researchers with little usable material from which to measure the carbon-14-tagged hormone precursors that the elephant has ingested. AMS comes to the rescue again.

Science in Revolution

For all this work to date, biological AMS is still very new. Knezovich acknowledges this, saying, "We at Livermore are still doing lots of missionary work, educating the research community about this powerful technology. But the NIH grant makes clear that the biological research community is convinced of the worth of AMS."

Livermore's AMS experts are part of an apparent revolution in which biology may be replacing physics as the haute discipline. Research in biosciences now accounts for more than 40 percent of federal funding for basic research, fueled in part by an aging population and increased needs for health care.

As the use of AMS for biological research grows and matures, related applications will quickly be found. CAMS has already participated in a study of the effects of atrazine, a commonly used herbicide, on a group of volunteers in California who wore a skin patch of atrazine for 24 hours. Radioactivity in their urine was too low to be counted efficiently with liquid scintillation counting. But with AMS, uptake and elimination could be followed easily. Samples were even chromatographically separated to determine how the subjects metabolized the compound during exposure, a day after exposure, and several days after exposure. These biomarkers provide the data needed to develop an assay for occupational exposure to this chemical.

From agriculture to nutrition, from toxicology to chemotherapy—the potential uses of AMS for our better health are almost endless.

—Katie Walter

Key Words: biomedical research, carbon-14, Center for Accelerator Mass Spectrometry (CAMS), Marshall Islands, MeIQx, National Institutes of Health (NIH), nutrition, pesticides, PhIP, plutonium, radioisotope tracing, tritium.

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About the Scientist

JOHN KNEZOVICH has been the director of the Center for Accelerator Mass Spectrometry since May 1998. He received his B.A. in biology from the University of the Pacific in 1977 and his Ph.D. in chemical ecology from the University of California at Davis in 1983. He was previously a group leader for Environmental Chemistry and Toxicology. Knezovich is an environmental chemist with extensive experience in the design and application of experimental approaches for determining the fate, transport, and toxicity of contaminants in the environment. He originally came to Lawrence Livermore as a student guest in 1977 and returned as a postdoctoral scientist in 1983. Knezovich serves on University of California, state, and federal advisory panels that oversee research on toxic substances. He has written more than 60 scholarly articles and publications.

Reducing the Threat of Biological Weapons

Livermore's strategy for defense against the use of biological weapons integrates technology, operations, and policy and provides a framework for coordinated local, state, and federal emergency response.

"WEAPONS of mass destruction" is a terrifying term. We all have mental images of the horrors of a nuclear attack, and photos of Kurdish and Iranian casualties of Iraqi chemical attacks attest to the devastation of chemical weapons. The third weapon of mass destruction—the biological weapon—has been around at least since the Middle Ages when soldiers catapulted the bodies of dead smallpox victims over fortress walls in the hope of infecting their enemies or at least demoralizing them.

Lately, biological weapons have been appearing in the news with increasing frequency. The anthrax threat in Las Vegas in February of this year is an example. Surplus stores in Las Vegas sold out of gas masks, and talk-radio shows were swamped with callers asking about evacuation points. That threat turned out to be a false alarm, but the next one might be real.

Biological agents are of concern in part because of the ease with which many of them can be manufactured, transported, and dispensed. And because of the lag time between a biological attack and the appearance of symptoms in those exposed, biological weapons could be devastating. Many biological agents are contagious, and during this lag time, infected persons could continue to spread the disease, further increasing its reach. Hundreds or even thousands of people could become sick or die if a biological attack were to occur in a major metropolitan area.

With the knowledge that several nations have produced and perhaps also deployed biological warfare agents, Congress in 1996 passed the Defense Against Weapons of Mass Destruction Act, which authorizes the Department of Energy to establish a Chemical and Biological Weapons Nonproliferation Program. Under this and similar programs, Lawrence Livermore and other laboratories and institutions are working together to increase this country's capabilities to detect and respond to an attack by biological or chemical weapons.

Beginning as recently as Fiscal Year 1996 with a Laboratory Directed Research and Development strategic initiative, Livermore has rapidly expanded its chemical and biological nonproliferation program and is now playing a lead role in this effort, particularly as it pertains to defense against biological weapons. The Laboratory is applying its investment in biological science, engineering, microtechnology, computer modeling, systems analysis, and atmospheric science to a number of programs designed to improve the country's response to a biological attack. Personnel from departments and directorates across the Laboratory are at work on:

- Advanced detection systems to provide early warning, identify populations at risk and contaminated areas, and facilitate prompt treatment.
- Biological forensics technologies to identify the agent, its geographical origin, and/or the initial source of infection.
- Methods for predicting the transport

of biological agents in urban environments and for assessing the area and duration of the hazards associated with a biological attack.

• New decontamination technologies to clean and restore facilities without causing further environmental damage.

Livermore is working closely with the U.S. military, various government agencies, and such major cities as New York City and Los Angeles to ensure that the results of these biological nonproliferation efforts meet the needs of military troops, the FBI, local law enforcement personnel, fire fighters, public health officials, and others who would likely be first on the scene following a biological attack. Together these groups are answering questions to help create the best, most task-appropriate, and most usable system possible. For example, how accurate do sensors have to be? What level of false alarms can be tolerated? Where will sensors be located—in buildings, on emergency response personnel, or at other sites? How much training will be feasible for emergency response personnel on the use of sensors and decontamination agents—that is, how user-friendly must these processes be?

Livermore is developing a strategy for defense against the use of biological weapons that integrates technology, operations, and policy and provides a framework for coordinated local, state, and federal emergency response.

Better Detection Systems

A key factor limiting the nation's ability to protect against a biological attack has been the state of biodetector technology. Only now is technology becoming available that permits identification of biological organisms within minutes, when concentrations are low but often still dangerous. Before the revolutions in genomics, biotechnology, microengineering, and microcomputers, such identification could only be done in a laboratory and took days to weeks. Soon, however, technology advances—many of them made at Lawrence Livermore—will offer the possibility of rapid, accurate, and sensitive biodetectors for use in battlefield or urban settings.

Figure 1. Ray Mariella, Jr., working with a multichambered PCR (polymerase chain reaction) unit. In the 1997 Advanced Concept Technology Demonstration, this PCR instrument proved an effective tool for field identification of the DNA in nonvirulent bioagent simulants.

Automation Is Key

Livermore is developing two types of fully automated biodetectors for real-time sample collection, detection, and identification in the field. A miniature flow cytometer (known as miniFlo) uses an immunoassay system to look at the proteins and other material on the surface of cells, and a portable PCR (polymerase chain reaction) unit identifies the DNA inside the cell. (See the box on p. 6 for more information on these systems.) Because of their small size and efficiency, both units process data much faster than their laboratory-scale cousins, while maintaining the highest level of sensitivity.

To fully automate sample collection and preparation, Livermore is developing and testing components for an aerosol biocollector and a microfluidic sample preparation system. The device will collect and sample particles in the air, including biological agents, if present. To maximize detection potential and give faster results, the PCR unit and miniFlo are also being "multiplexed" to handle multiple samples at once. Other system improvements are being made to both instruments to lower the rate of false positives (false alarms), increase the sensors' sensitivity, and make the systems even smaller, more rugged, and less reliant on consumables than they are now. Livermore expects to have continuously operating, integrated biosensors available for use within the next few years.

With two types of sensors working in tandem, the chance of false alarms will be reduced considerably. Tolerance for false alarms differs greatly for military versus civilian situations. Deployed troops are already in a state of heightened readiness, with protective equipment available and the training required to react to attack situations. In contrast, with civilians, false alarms could lead to injuries and perhaps to dismissal of future legitimate alarms. Thus the military may be able to afford some level of false alarms, but the goal for the civilian sector is no false alarms.

The miniFlo and the PCR systems have proved their mettle against established performance criteria at the U.S. Army's international Joint Field Trials at the Dugway Proving Grounds in Utah. At Dugway, participants use a variety of instruments to detect simulant materials representative of typical biological weapon materials.

At the 1996 Joint Field Trials III, miniFlo was superb at detecting *Bacillus globigii* and *Erwinia herbicola* (nontoxic simulants for anthrax and plague respectively) at various low concentrations. Overall, miniFlo detected 87% of all unknowns with a false alarm rate of under 0.5%. At the 1997 Port/Airbase Advanced Concept Technology Demonstration and the January 1998 Dugway Joint Field Trials IV, the portable PCR unit clearly demonstrated the potential of PCR as an effective technique for field identification of DNA (Figure 1).

Networked Detectors

A networked system of these or other biodetectors could provide U.S. troops in the field with early warning of a biological attack. That is the goal of a project for the Department of Defense known as JBREWS (Joint Biological Remote Early Warning System), on which Livermore is collaborating with Johns Hopkins Applied Physics Laboratory and Los Alamos National Laboratory. As shown in Figure 2, JBREWS will consist of a network of sensors and communication links. By tying this network into the military's existing communications systems, JBREWS will take advantage of well-established command and communications procedures. Initially equipped with commercially available sensors, JBREWS is being configured so that improved biodetectors can be incorporated into the system as they become available.

Livermore is responsible for what is known as "C4I"—command, control, communications, computers, and intelligence. The Laboratory is developing the connectivity between the sensors and the control station, the software for all sensors, and an automatic analysis and reporting system that runs up through the military chain of command. JBREWS is scheduled to be demonstrated in a Department of Defense Advanced Concept Technology Demonstration in 1998.

Biological Forensics at Work

If a bacterium or spore appears in a collected sample, how will a biodetector know what it is? The key to identification will be a library of "signatures" of the makeup, function, and DNA of various biological agents that will be stored on a microchip in the detector, together with pattern-matching software and code for reporting results. This technology will allow advanced detectors in the laboratory and ultimately in the field to quickly match the signatures of collected particles to signatures in its memory, in much the same way that fingerprints are matched.

Building on years of experience in genomics and biotechnology, Livermore scientists are expanding the

Livermore's New Biodetectors

Portable PCR

In late 1996, Lawrence Livermore delivered to the U.S. Army the first fully portable, battery-powered, real-time DNA analysis system. DNA analysis requires many copies of a DNA sample, which are made by the polymerase chain reaction. PCR requires repeated cycles of an aqueous sample being heated close to the boiling point and then cooled. To detect DNA in a sample, a synthesized DNA probe or primer tagged with a fluorescent dye is introduced into the sample before it is inserted into the heater chamber. Each probe or primer is designed to attach to a specific organism—anthrax, plague, etc. If that organism is present in the sample, the probe attaches to its DNA. By measuring the sample's fluorescence, the instrument reports the presence (or absence) of the targeted organism.

In Livermore's portable unit, the thermal cycling process takes place in a micromachined, silicon heater chamber that has integrated heaters, cooling surfaces, and windows through which detection takes place. The PCR reaction and DNA analysis take place in a disposable polypropylene reaction tube inserted into the heater chamber.

Because of the low thermal mass and integrated nature of Livermore's silicon heater chambers, they require very low power and can be heated and cooled much faster than conventional units. So the unit is not only portable but also much faster and more energy-efficient than bench-top models. A multiple-chamber unit that allows the examination of many samples at the same time has been field tested.

MiniFlo

Livermore's miniature flow cytometer is the latest in a series of flow cytometers developed over the past two decades in Livermore's Biology and Biotechnology Research Program Directorate. Flow cytometers are used in laboratories to analyze cells and their features, perform blood typing, test for diseases and viruses, and separate out particular cells or chromosomes. What sets miniFlo apart from other flow cytometers is its small size, portability, and sensitivity.

These features are made possible by a novel system that eases the alignment and increases the accuracy of flow cytometry. In a flow cytometer, the cells flow in single file in solution while the experimenter directs one or more beams of laser light at them and observes the scattered light, which is caused by variations in the cells or DNA. Instead of using a microscope lens or an externally positioned optical fiber as a detector, this method uses the flow stream itself as a waveguide for the laser light, capturing the light and transmitting it to an optical detector. This approach not only eliminates the alignment problems that plague traditional flow cytometers but also collects ten times more light than a microscope lens does. Simpler alignment and more light mean better, faster analysis.

Bacteria are large enough for individual detection in the miniFlo, but viruses and proteins are not. So beads large enough to be detected are coated with an antibody and added to the sample. The virus or protein attaches itself to the bead and can then be detected. When different beads are coated with different antibodies, simultaneous detection of several biological agents is possible.

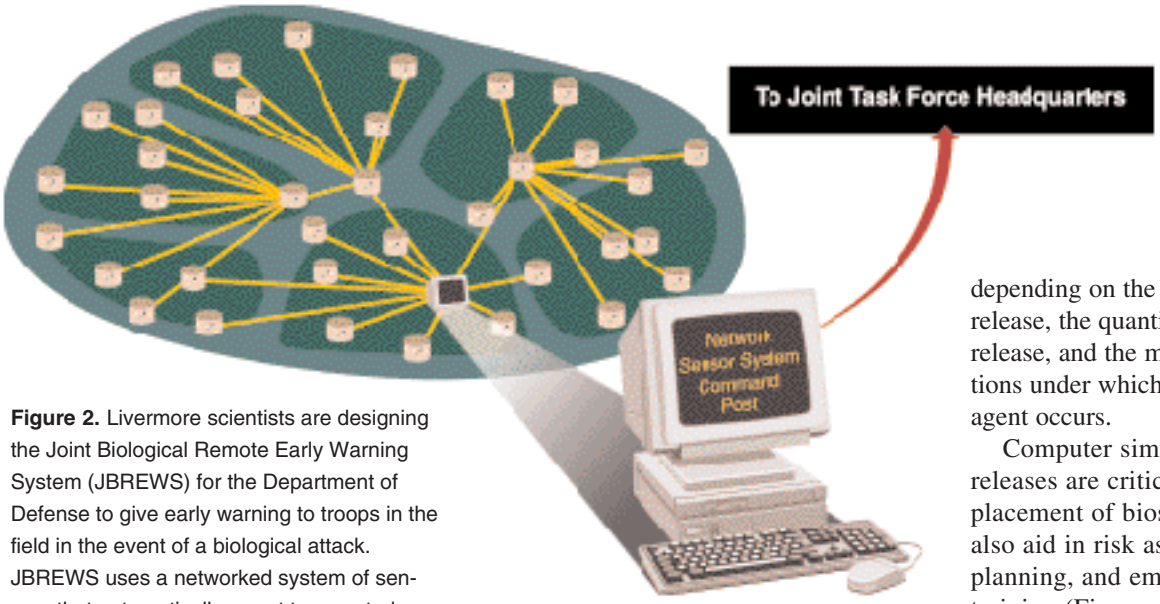

Figure 2. Livermore scientists are designing the Joint Biological Remote Early Warning System (JBREWS) for the Department of Defense to give early warning to troops in the field in the event of a biological attack. JBREWS uses a networked system of sensors that automatically report to a central computerized command post.

information base of the DNA sequences of biological agents to enable rapid, unambiguous identification of biological agents. To facilitate this process, they are developing ways to speed up the process of finding unique DNA sequences among organisms.

A process known as representational difference analysis helps to identify unique DNA sequences. Parts of the DNA of two organisms are mixed. If they stick together, they match; if they do not stick, they are unique parts. Currently, this process is cumbersome and slow, but Livermore scientists are working to automate it to be able to examine many sequences in parallel.

Another project is studying specific pieces of bacterial DNA and examining the possibility of using their location as an indicator of differences among strains. A third project is investigating virulence factors, which are the genes that give a biological organism its infectivity or toxicity. If a bioweapon is being genetically engineered, those genes might be moved to an unnatural host in an attempt to thwart detection and identification.

In addition to identifying the particular agent being used, tools being developed at Livermore also seek to provide information that will help to identify the perpetrator of a biological attack. Livermore biomedical researchers were among the first to study regional differences among the various naturally occurring strains of anthrax and other biological agents. Law enforcement personnel will be able to match data about a pathogen with data on regional or strain characteristics (indicators of engineered characteristics) and with data on worldwide biological research, epidemiology, and infectious diseases and respond to the threat.

Predicting Agent Dispersion

The ability to accurately predict the dispersion, concentration, and ultimate fate of biological agents released into the environment is essential to prepare for and respond to a biological agent release. Of particular concern is the threat to civilian populations within major urban areas where potential terrorist incidents are more likely to occur. There the hazard from a biological-agent release could be confined to a localized area within or around a single building or extend out to a large portion of the city or even into the surrounding suburbs, depending on the particular agent release, the quantity and duration of the release, and the meteorological conditions under which dispersion of the agent occurs.

Computer simulations of biological releases are critical to the design and placement of biosensor systems. They also aid in risk assessment, disaster planning, and emergency response training (Figures 3 and 4). If a biological release were to occur, real-time predictions of agent concentrations would be used to characterize the source, estimate exposure levels, identify affected areas and best evacuation routes, and later assist with decontamination. Accurate information about the likely course of a bioagent attack is key for emergency response managers, who must notify health officials, inform emergency response teams, and make public safety decisions.

The urban biological release problem is quite complex and requires modeling capabilities that are still in the early stages of development and application. For example, models of airflow inside buildings and subways have been developed to some degree but do not accurately incorporate the decrease in airborne concentration that results from deposition of the toxic material on walls, ceilings, ventilation ducts, and other interior surfaces. Similarly, computational fluid dynamics models of the highly distorted flows and dispersion patterns created by complexes of buildings are just beginning to include the effects of biological aerosols (gravitational settling, deposition, and viability degradation) and multiple building interactions.

Lawrence Livermore, Lawrence

Berkeley, Los Alamos, and Argonne national laboratories are working together to develop an integrated and validated atmospheric modeling capability for biological agent releases in an urban environment. They will be applying these models to case studies in a range of release scenarios, from closed office buildings, to subway systems, to stadiums and street corners. The goal is to make the models applicable to real-life situations and ultimately to integrate them into the incident response capability of the National Atmospheric Release Advisory Center, located at and operated by Lawrence Livermore.

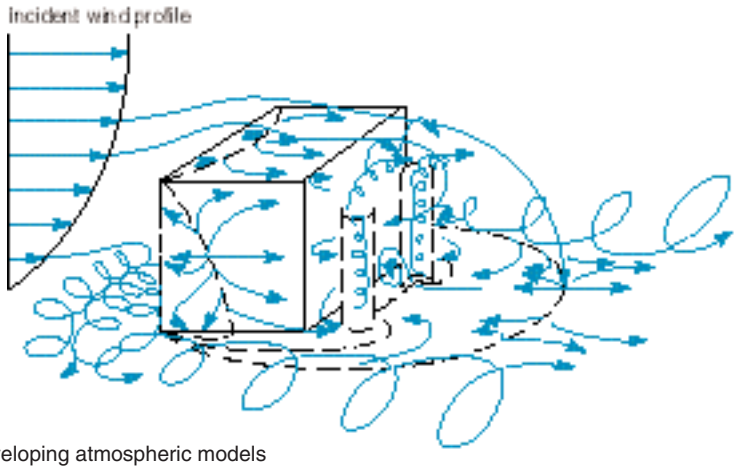

Figure 3. Developing atmospheric models for an urban setting requires taking many flow patterns into consideration. As shown here, air movement around just one building is highly complex.

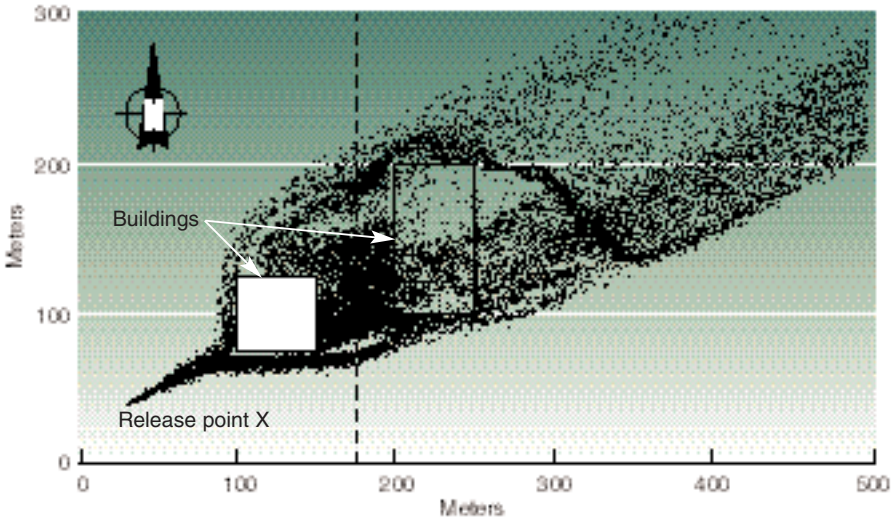

Figure 4. This scenario shows where particles will be 10 minutes after they are released at point X in a 240-degree (west southwest) wind of 10 meters per second. Several areas of high particle concentration are visible to the south of the two buildings, with lesser concentrations to the north and to the east.

Decontaminating a Site

After an area has been exposed to a biological attack, it must be decontaminated before it can be reopened to the public. Livermore and Los Alamos national laboratories are working together to develop decontamination strategies for three scenarios—an open stadium, a semi-enclosed subway, and an enclosed area such as an office or home. Certain decontamination methods might be acceptable for one scenario but not another. For example, more corrosive reagents and large volumes of water might be acceptable in a stadium but could not be used in an office building.

Plain household bleach is one of the best decontamination agents around, and it is used regularly in biological laboratories throughout the country. But 5% sodium hypochlorite (as bleach is more technically known) is a very caustic product, so it must be used with care. The team is working to develop decontamination methods that are as effective as bleach but more acceptable environmentally.

Decontamination proceeds in several stages, from cleanup of gross contamination such as puddles of agent, to localized decontamination of walls or furniture that were directly exposed to the agent, to cleanup of ductwork or inaccessible cracks for hidden contamination, and finally to long-term remediation such as special paints or sorbents to destroy small quantities of agent that are left after completion of other decontamination. These stages may require different cleanup materials. A variety of liquids and powders are being studied, as is an array of delivery methods such as foams and gels. One treatment method that has been found to be effective and more environmentally acceptable than hypochlorite (an alkaline product) is peroxymonosulfate, which is an acidic oxidizer. Figure 5 compares treatment

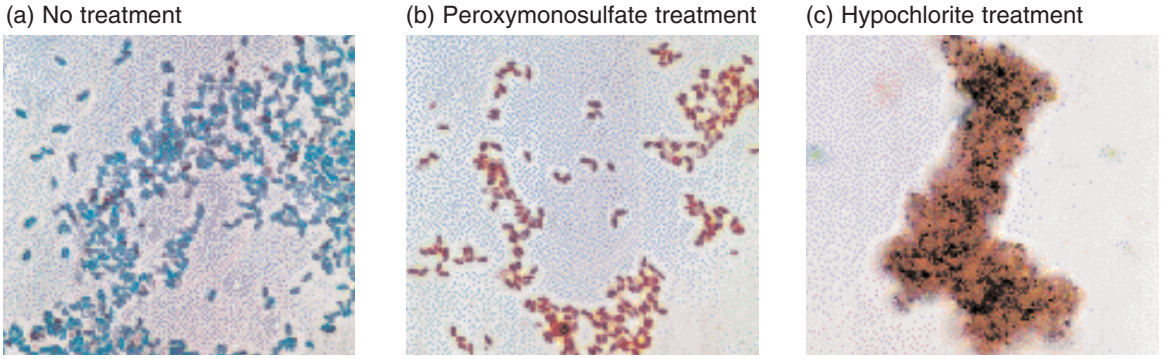

Figure 5. *Bacillus globigii* spores (a simulant of the spores that cause anthrax) are shown (a) before and (b) after a 30-minute exposure at 22°C to peroxymonosulfate, an acid oxidizer, and (c) after treatment with hypochlorite, an alkaline oxidizer. Spores were stained with malachite green (blue-green) and safranin (red) dyes. Safranin dye penetrates only dead spores because of their damaged walls, thus making it a good indicator of the effectiveness of a biocide.

of a simulant for anthrax with these oxides. The selected method must be not only effective but also easy to use with minimal training.

The social and political issues involved in decontamination and reentry to a site are not being overlooked. Central to these concerns is "How clean is clean enough?" The team is coordinating with the biosensor developers to devise sampling and analysis systems that can verify that decontamination is complete.

One hurdle for the decontamination process is that no real-time biodetector currently under development at Livermore uses an assay that can distinguish between viable organisms and dead or decontaminated ones. Work has begun on a "viability assay" based on flow cytometry to provide this important piece of information so that decontamination can proceed in a timely manner.

Responding to the Threat

The threat of biological weapons is all too real, and the U.S. must be prepared to respond if a bioattack occurs on the battlefield or in a civilian setting. During the 1991 Gulf War, the U.S. had no systems available for rapid, timely field detection of bioagents. The situation today is very different. The military has deployed Biological Integrated Detection Systems (BIDS), which can tentatively identify the presence of a suspected biological agent in the field and warn soldiers to take appropriate action to protect themselves against the agent, pending positive laboratory identification. And there are also programs such as Livermore's that include new detection, identification, atmospheric modeling, and decontamination capabilities, which, combined with work by others on better vaccines and medical treatment, are bringing the country to a level of preparedness that can meet a biological threat.

—Katie Walter

Key Words: biodetectors, bioinformatics, biological warfare agents, decontamination, DNA analysis, flow cytometry, genomics, miniFlo cytometer, National Atmospheric Release Advisory Center (NARAC), polymerase chain reaction (PCR), weapons of mass destruction (WMD).

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FRED MILANOVICH received his B.S. in physics from the United States Air Force Academy in 1967 and a Ph.D. in applied physics from the University of California at Davis in 1974. He is currently program manager for the Chemical/Biological Nonproliferation Program within the Nonproliferation, Arms Control, and International Security Directorate at the Laboratory. This program is providing an integrated response to the emerging threat of chemical and biological terrorism with innovation in detection technology, bioinformatics, fate and transport analyses, and incident response. Milanovich has published extensively in his field and holds many patents for optical sensors and measurement instruments. His research interests also include trace biodetection, laser spectroscopy, analytical instrumentation development, and microtechnology.

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Science & Technology Review
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